Systematics and Phylogeography of Seychelles Amphibians

Simon T. Maddock

University College London

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Supervisors: Dr Julia J. Day (UCL) & Dr David J. Gower (NHM)

ABSTRACT

This thesis investigates evolutionary patterns of variation in endemic amphibians from the Seychelles archipelago. Focal groups include the treefrog (*Tachycnemis seychellensis*), and a radiation of caecilians in three genera (*Grandisonia, Hypogeophis* and *Praslinia*), and attempts to place these into a phylogenetic context. The introduction (Chapter 1) discusses the importance of islands in the study of evolution and examines patterns of intraspecific variation that have been reported in other Seychelles organisms. Chapter 2 provides the first intraspecific molecular study of the monotypic Seychelles treefrog *Tachycnemis*, implementing a species tree approach in order to investigate its relationship with its closest living relatives (*Heterixalus*) from Madagascar and test whether its ancestor colonised the Seychelles via overseas dispersal. Chapters 3 and 4 explore variation in the six species of Seychelles caecilian, all of which overlap in range on at least one island. To assess within- and among-island intraspecific variation in these subterranean amphibians, Chapter 3 uses genetic data from both mitochondrial and nuclear markers, while Chapter 4 uses morphometric and meristic data. Differing patterns of geographic structure was observed among the caecilian species. The final two data chapters analyse species-level relationships among the Seychelles caecilians. Chapter 5 utilises Next Generation Sequencing to obtain mitogenomic data, and multiple approaches to infer phylogeny, and the effectiveness of alternative methods are evaluated. Chapter 6 attempts to resolve relationships of the island caecilians using 11 nuclear loci and multiple methods of phylogenetic inference. Chapter 7 discusses how the thesis has

increased knowledge of the study taxa and of the evolution of amphibians on islands, particularly the Seychelles.

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CHAPTER 1

INTRODUCTION

Island systems provide good opportunities to study patterns of evolution because of their often extreme isolation and restrictive boundaries. These factors combined imply that very little migration is likely to occur and selection pressures may therefore elevate speciation. Age, relative seclusion and size of islands all contribute to evolutionary patterns observed on island systems (e.g. Emerson and Oromí, 2005; Gillespie and Roderick, 2002; Gillespie et al., 2008; Grant, 1998; Losos and Parent, 2009; Parent et al., 2008; Rabosky and Glor, 2010). The main causation of speciation is that of physical separation (Mayr 1942) (however, see for example Mayr 1984), and it is therefore unsurprising that island systems generally have a high proportion of endemic species. To date most studies examining island evolution of animals have focussed on lizards and birds while other faunal groups such as amphibians have been comparatively neglected.

Robert MacArthur and Edward Wilson prompted the field of island biogeography in 1967 with their landmark book *The Theory of Island Biogeography*. Their main focus investigated species equilibria on islands using immigration and extinction curves (MacArthur & Wilson 1963), island size, isolation, and evolution; most of their theories were later confirmed by one major experiment (Simberloff & Wilson 1971). Since these original works, the field has grown exponentially and the theory modified accordingly. Recently other workers have pushed for an expansion of and a more integrated approach to the theory (see Losos & Ricklefs 2010).

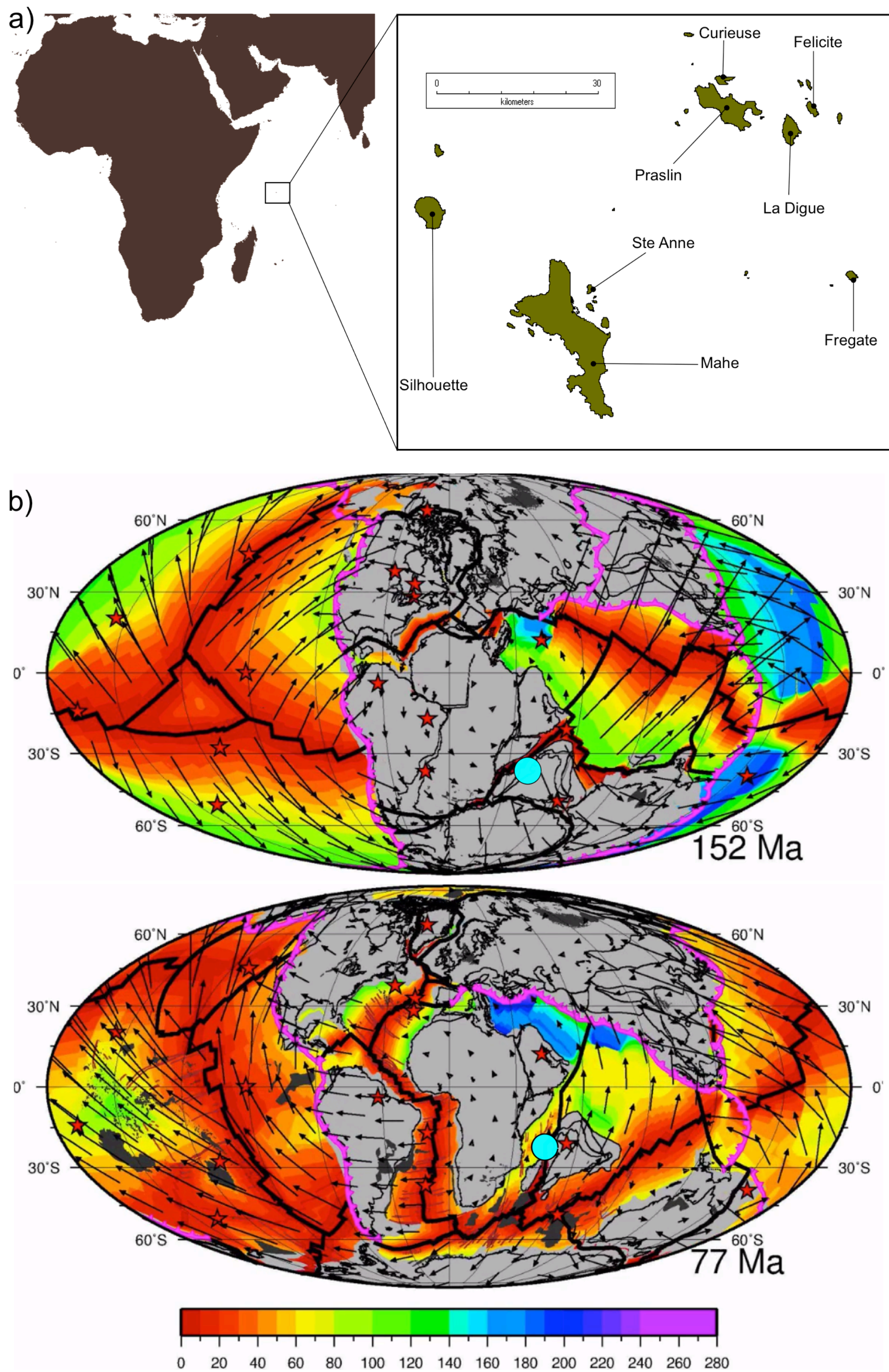

Fig 1. a) Map showing the positioning of the Seychelles and the main granitic islands discussed in this thesis. b) Two global reconstructions showing the breakup of Gondwana during different time periods. Turquoise circles represent the approximate positioning of "Seychellea". Gondwana reconstructions taken from videos of Seton et al. (2012).

Fig 2. a) Map showing the main granitic islands of the Seychelles discussed in this thesis.

Seychelles palaeogeography

The Seychelles archipelago consists of 42 granitic and 113 coralline islands stretching between 4-11°S and 45-56°E in the western Indian Ocean. The granitic islands are the furthest north and are considerably older than the coralline islands, having formed part of the now fragmented ancient continent of Gondwanaland (Fig. 1). Approximately 175 Ma (Schettino & Scotese 2005) a landmass consisting of Madagascar, India, Seychelles, Antarctica and Australia drifted south-south-east from the rest of Gondwanaland (Coffin & Rabinowitz 1987; Jokat 2003; Rabinowitz & Woods 2006) before Madagascar reached its present day position after moving along the Davie Fracture Line approximately 16 Ma (Schettino & Scotese 2005). Up until 90-95 Ma the Kerguelen Plateau (Coffin 1992; Frey *et al.* 2000; Mohr *et al.* 2002; Ali &

Aitchison 2009) would have created a connection between the Seychelles, Madagascar and India together (Ali & Aitchison 2008).

By 83.5 Ma it is likely that a large area of ocean would have separated India/Seychelles and Madagascar as a result of India's northwards movement towards Asia (Ali & Aitchison 2008). At approximately 61 – 65 Ma the formation of the Carlsberg Spreading Ridge would have completely separated the Seychelles from India (Chatterjee et al., 2013; Collier et al., 2008; Davies, 1968; Dickin et al., 1986; Mart, 1988; McElhinny, 1970; McKenzie and Sclater, 1971; Norton & Sclater 1979). The formation of the Carlsberg Ridge succeeded by the opening of the Gop Rift (Collier *et al.* 2008) although its age is debated (e.g. Armitage et al., 2011; Eagles and Hoang, 2013; Yatheesh et al., 2009).

The granitic islands of the Seychelles are part of the emergent parts of the microcontinent "Seychellea", which has a total area of 129,650km^2 and which currently has an average depth of 55m below present sea level (bpsl) (Davies & Francis 1964). The currently emergent granitic islands are the highest 'mountains' of Seychellea and contain the largest islands of the Seychelles Archipelago: Mahé (142km^2), Silhouette (20km^2), Praslin (38km^2) and La Digue (10km^2). Of this group the largest southern islands of Mahé and Silhouette are also the highest, reaching 914m/asl and 740m/asl, respectively. The habitat of these southern granitic islands are different to those further north, having middle elevation wet forests and high elevation moss forests as well as typical lower elevation habitats found throughout the archipelago (Vesey-Fitzgerald 1940) (see Fig. 3 for representative habitat pictures). The continental, not volcanic, history of the Seychelles makes the diversity of the

biota interesting because it contains a mix of ancient endemics of Gondwana and more recent transoceanic arrivals.

Fig. 3. Examples of habitats across the granitic Seychelles (photos by S. Maddock).

Fluctuations in global sea-level are controlled primarily by the melting and refreezing of the polar ice caps, principally associated with ice ages and interglacial periods (e.g. see Fleming et al. 1998; Yokoyama et al. 2000, 2001). Other factors such as tectonic movements, and isostatic and hydrostatic changes can also influence sea levels (Camoin et al. 2004).

Eustatic sea level fluctuations around the world have been great, with low stands lower than 120m bpsl estimated to have occurred, multiple times, within the last 2 Ma and with even more drops below 50m within the last 4 Ma (Miller et al. 2005). Several episodes of low stands in sea level occurred in the Indian Ocean within the last 500ka, matching world-wide estimates (Camoin et

al. 2004; Colonna et al. 1996; Rohling et al. 1998; Siddall et al. 2003) with estimates of low stands down to 145 ± 5m bpsl reported (Camoin et al. 2004). These low stands would have reconnected all of the main granitic islands (Fig. 4). As well as low stands there have been multiple instances of high stands, although these were not as prevalent within the last 30 Ma (Miller et al. 2005). At the last interglacial (~125,000 Ka) it is likely that sea levels around the Seychelles could have risen by 5.5m to 9.4m (Dutton & Lambeck 2012; Kopp et al. 2009; Israelson & Wohlfarth 1999), other estimates have placed global levels much higher (e.g. Miller et al. 2005). It is likely that with these increases in sea level many of the coralline islands of the Seychelles would have become fully submerged (e.g. Thomson & Walton 1972). Although the granitic islands would have generally remained above water some of the islands would have significantly reduced in size (Nussbaum & Wu 1995). Cycles of island dis- and reconnection can be predicted to have had a substantial impact on gene flow in Seychelles organisms, and thus on their evolution.

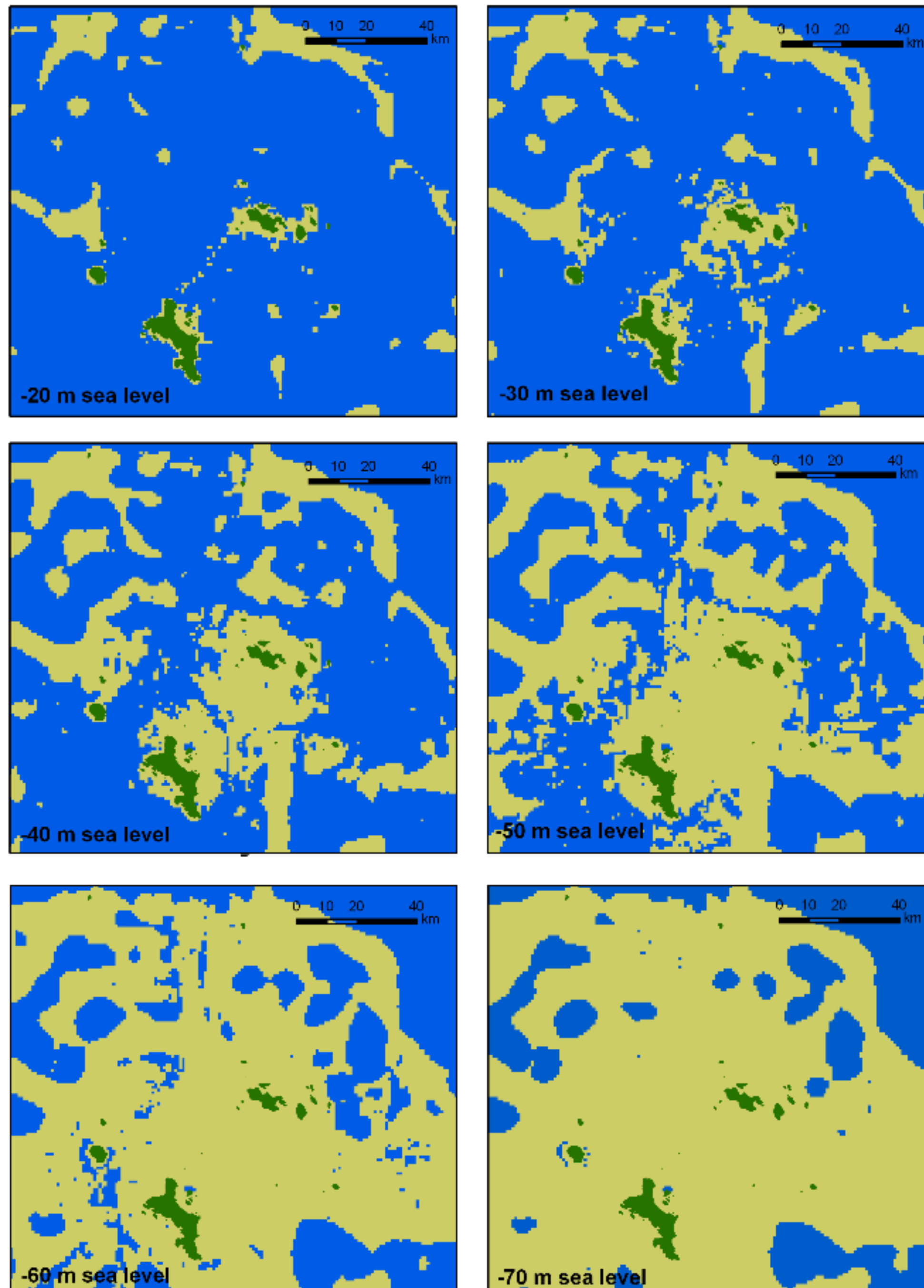

Fig. 4. Maps showing levels of connection between the granitic islands during low stands in sea level. Modified from Rocha et al. (2013).

Evolutionary patterns within the Seychelles

In contrast to other island systems (e.g. Galapagos, Canary and Hawaiian islands), the Seychelles has been largely overlooked regarding studies investigating intra-archipelago biotic assembly, variation and diversification.

Only recently has an increased effort been made to document patterns of evolution and diversity using detailed sampling of molecular (Daniels, 2011; Legrand et al., 2009; Palkovacs et al., 2003; Radtkey, 1996; Rocha et al., 2013, 2011, 2010; Silva et al., 2010a; Taylor et al., 2012; Valente et al., 2014; Van Der Meijden et al., 2007) and morphological (Scott 1933; Nussbaum 1984a, 1984b; Cheke 1984; Gardener 1987; Nussbaum & Wu 1995; Radtkey 1996; Gerlach 1999; Gerlach & Bruggen 1999) variation.

Many Seychelles taxa (e.g. birds, lizards, frogs, crabs, spiders) have been included in molecular analyses, but principally in order to investigate timing and origin of colonisation or to place them within a higher-level phylogeny. An emergent pattern regarding colonisation of the Seychelles is that taxa that arrived more recently via transoceanic arrival generally have an African origin (e.g. Guo et al., 2012; Townsend et al., 2011; Vences et al., 2004, 2003; Wollenberg et al., 2007), whereas those that are more ancient generally have oriental affinities caused by vicariance when Seychelles split from India (Biju and Bossuyt, 2003; Gower et al., 2011; San Mauro et al., 2014).

Patterns of intraspecific variation among the Seychelles biota has uncovered contrasting patterns of geographic structure among organisms. The most studied organismal group to date are lizards, this includes two lineages of gecko and two lineages of skink (Radtkey 1996; Rocha *et al.* 2010a, 2011, 2013a; Valente *et al.* 2014). These studies of Seychelles lizards have uncovered a consistent geographic split between specimens from northern and from southern islands. Lizards from the easternmost granitic island of Frégate share a closer affinity with specimens from the southern islands for most species except for the gecko *Phelsuma astriata* (in species tree analysis)

(Rocha *et al.* 2013a) and the skink *Pamelaescincus gardineri* (Valente *et al.* 2014) for which Frégate specimens share a closer affinity with specimens from the northern islands. A similar pattern to that observed in lizards has been reported in *Drosophila* flies, which also exhibit northern and southern lineages (Legrand *et al.* 2011). Other organisms show intraspecific geographic structuring across all of the island populations that they are distributed on (freshwater crabs: Daniels, 2011; sooglossid frogs: Taylor et al., 2012; Van Der Meijden et al., 2007) and island populations (Seychelles treefrog: Nussbaum and Wu, 1995), though it should be noted that the treefrog and crab have a more restricted distribution than the lizards. The testudines show no geographic structuring across the entirity of their range (giant tortoises: Palkovacs et al., 2003; freswater turtles: Silva et al., 2010b).

The Gondwanan history of the Seychelles means that the biota is composed of a mix of ancient endemics and more recent transoceanic arrivals. These ancient endemics are believed to have been isolated on the Seychelles for at least 64 Ma since India and Seychelles separated (Biju & Bossuyt 2003; Roelants *et al.* 2007; Gower *et al.* 2011; San Mauro *et al.* 2014); suggesting that vicariance is to explain for the ancient fauna seen on the islands.

Contradictory however to the hypothesis that ancient endemics are of Indian origin, the two ancient (and sister genera) Seychelles scincine lizards, *Janetaescincus* and *Pamelaescincus*, may represent the sister taxa to Malagasy-African scincines (Brandley *et al.* 2005; Pyron *et al.* 2013) suggesting an ancient Malagasy-African origin (but relationships with Indian and Sri Lankan scincines are poorly known); this could therefore suggest that

vicariance may have had a large role in the development of faunal assemblages early on in the breakup of Gondwana. It is possible that ancient endemics such as the scincines with a seemingly more Malagasy-African origin could have colonised via the Kerguelen Plateau, a now-submerged region in the south east Indian Ocean (Coffin 1992; Frey *et al.* 2000; Mohr *et al.* 2002; Ali & Aitchison 2009). The Keguelen Plateau currently lies approximately 1km below the surface of the ocean (with a few emergent islands still observable) but until 90-95 Ma it linked Seychelles, Madagascar and India together (Ali & Aitchison 2008), allowing for a possible interchange of fauna between landmasses up until this point.

Most terrestrial vertebrates on the granitic Seychelles are believed to have colonised the islands via transoceanic arrival once the Seychelles was fully isolated from other landmasses. Although relatively few studies have estimated colonisation times or dispersal routes for the Seychelles biota, colonisation is believed to be primarily from South Africa (29-49 Ma) (Townsend *et al.* 2011), east Africa (16 Ma) (Guo *et al.* 2012), or Madagascar (10-35 Ma) (Vences *et al.* 2003b; Crottini *et al.* 2012) rather than from India. Until further work is carried out then transoceanic arrival from India cannot be ruled out for vertebrates.

Seychelles amphibians

Amphibians are a relatively rare phenomenon on islands (e.g. Vences et al., 2003) and islands that do have a native amphibian fauna tend to be close to a mainland. Amphibian skin is porous and is susceptible to osmotic forces (e.g. Balinsky, 1981; Duellman and Trueb, 1986) that restricts dispersal ability over

saltwater and this is a causative factor in the low numbers of amphibian species observed on small islands (Bossuyt & Milinkovitch, 2001; Darwin, 1859; Myers, 1953, but see Measey et al., 2007; Vences et al., 2003, 2004). Compared to many other small island systems the Seychelles is relatively speciose in amphibians, comprising twelve species, all except one of which is endemic. This endemic fauna includes the only amphibian family to occur solely on islands (sooglossid frogs), a radiation of caecilians (Gymnophiona) consisting of six species in three genera (the only caecilian genera restricted to islands Nussbaum 1984a), and a hyperoliid treefrog that is a more recent colonist from Madagascar (Wollenberg *et al.* 2007). The non-endemic Mascarene rocket frog, *Ptychadena mascareniensis,* is considered to be introduced to the Seychelles because it is distinct from populations from Madagascar and the Mascarene islands (Vences *et al.* 2004).

Caecilian amphibians are one of the most poorly known of the major groups of vertebrates. The approximately 200 described species are mostly tropical and most spend their adult lives burrowing in moist soil, in water bodies, or decaying vegetation. They differ from members of the other major amphibian groups i.e. Anura (frogs/toads) and Caudata (salamanders/newts) in lacking limbs, and in having externally annulated bodies, substantially reduced eyes and a pair of sensory tentacles. Caecilians are identified as the sister group to the anurans + caudates (e.g. Pyron and Wiens, 2011; Roelants et al., 2007) and ten families are currently described (Wilkinson *et al.* 2011; Kamei *et al.* 2012) with all but one occurring on a single continent.

1.3.1 Seychelles treefrog, Tachycnemis seychellensis

The Seychelles tree frog, *Tachycnemis seychellensis,* is endemic and the only member of the genus*,* which is part of the largely African family of reed/tree frogs Hyperoliidae. Despite only occurring on four granitic islands of the Seychelles, it is considered common and adaptable, and is currently listed as "Least Concern" in the IUCN Red List. The species is restricted to areas surrounding water bodies up to an altitude of ~700m/asl (pers. obs.) in a range of habitats including high-elevation cloudforests and marshy plateaus. It is widespread on the two largest islands of Mahé and Praslin with few scattered populations on Silhouette and La Digue (Nussbaum & Wu 1995). Genetic data indicate that the closest relative of *T. seychellensis* is the Madagascan genus *Heterixalus*, and that the evolutionary split between *T. seychellensis* and its closest African/Madagascan relative occurred approximately 11 – 21 Ma (Vences et al. 2003). This divergence is too recent for the split between these taxa to have been caused by the separation of Seychelles and Madagascar. If the dating estimate is correct, treefrogs must have arrived in Seychelles via a rare oceanic dispersal. The Madagascan and Seychelles hyperoliid frogs form a monophyletic group nested within the African hyperoliid frogs (Richards & Moore 1996; Vences *et al.* 2003a; b; Frost *et al.* 2006; Pyron & Wiens 2011; Crottini *et al.* 2012). Though it cannot be ruled out that the Seychelles was colonised by the hyperoliids prior to Madagascar, through the examination of the diversity within Madagascar and palaeogeographic data for the region it is a well established belief that the ancestor of *T. seychellensis* colonised the Seychelles from Madagascar (e.g. see Vences et al. 2003).

Tachycnemis seychellensis has high levels of morphological variation between populations, both among islands and between two geographically close localities on the largest island of Mahé (Nussbaum & Wu 1995). Other authors have briefly reported differences in dorsal colouration between populations of the species (Schiøtz 2003). The high levels of geographic variation within the species, even within single islands (Nussbaum & Wu 1995), makes *T. seychellensis* an interesting study organism (see Fig. 5 for representative specimens). No other Seychelles organism has been discovered to have such high levels of morphological intraspecific variation as that observed in *T. seychellensis*, but to fully understand if this variation is explained by evolutionary history or by another factor such as phenotypic plasticity, detailed genetic work needs to be conducted on the species.

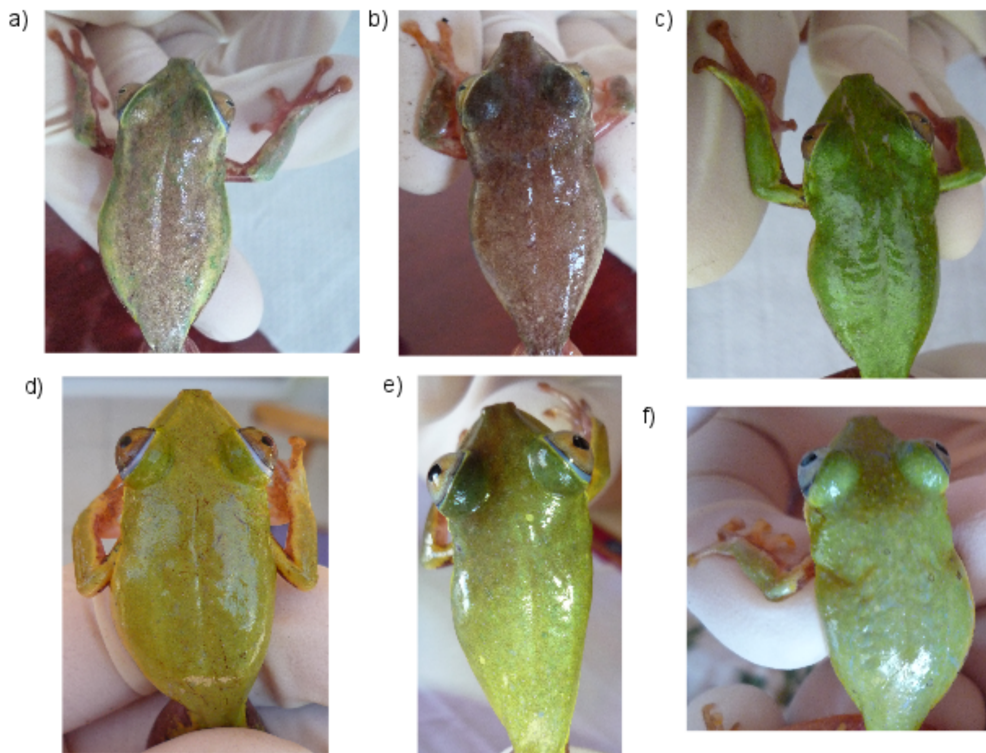

Fig. 5. Representative male specimens from across the range of *Tachycnemis seychellensis*: a) La Reserve, Mahé; b) Grand Bois River, Mahé; c) La Passe, Silhouette; d) Plateaux, La Digue; e) Anse Kerlan River, Praslin; f) Baie Saint Anne, Praslin (photos S. Maddock).

1.3.2 Seychelles caecilians

There are currently six recognised species of caecilian in three genera (*Grandisonia alternans, G. larvata, G. sechellensis, Hypogeophis brevis, H. rostratus, Praslinia cooperi,* see Fig. 6) from the Seychelles, all of which are endemic (e.g. Nussbaum 1984; Wilkinson et al. 2011) and which form a radiation that nests within the predominantly Indian (but also African) family Indotyphlidae (Wilkinson et al. 2011). Other than *H. brevis* (Endangered) and *P. cooperi* (Vulnerable), the Seychelles caecilians are listed as Least Concern in the IUCN Red List. These differences are based on one very simple consideration – breadth of distribution.

Though only one molecular study (San Mauro *et al.* 2014) has included an African indotyphlid in an analysis including Indian and Seychelles indotyphlids, they found that they (*Idiocranium*) formed the sister genus to the Seychelles and Indian species. It is possible that the Seychelles caecilians could have colonised from Africa or India via transoceanic dispersal but it seems unlikely given that caecilian dispersal over saltwater barriers is extremely rare. It is therefore commonly believed that the Seychelles caecilians were separated from their Indian counterparts during the separation of India and the Seychelles ~65 MA (Gower *et al.* 2011; San Mauro *et al.* 2014).

Previous morphological systematic work on the Seychelles caecilians involved the description of nominal taxa and documentation of variation in commonly assessed characters (e.g., numbers of annuli). The genera and species currently recognized were described between 1829 and 1968 (Taylor 1968; Wilkinson & Nussbaum 2006). The main areas of taxonomic

disagreement have been the generic assignment of *H. brevis* (formerly *G. brevis,* see Wilkinson et al. 2011), the validity of some previously described species e.g., *G. diminutiva* (Taylor 1968) currently considered a synonym of *G. sechellensis* (Boulenger, 1911) (Wilkinson & Nussbaum 2006), and whether different island populations of *H. rostratus* are distinct enough to merit subspecific status.

Beyond early life history strategies, many aspects of Seychelles caecilian ecology are poorly known. All of the Seychelles caecilians are carnivores and relatively similar in total size. The species can be found in moist soils and are strongly overlapping in vertical and horizontal distributions (but data is patchy and mostly unpublished).

Though some of the most extensive and intensive caecilian fieldwork has been carried out by R.A. Nussbaum on the Seychelles between 1976 – 1991, aspects such as niche partitioning, variation in diet, seasonality, demography, locomotion, and complete distributions remain unknown. Since R.A. Nussbaum carried out his fieldwork there has been very little caecilian work conducted on the group. Much of the natural history data collected by R.A. Nussbaum remains unpublished.

The Seychelles has played an important historical part in studies of the early life history and development of caecilians, based particularly on influential work of the German anatomists Brauer (1897, 1899), Marcus (1933), Eifertinger (1933), Marcus et al. (1933, 1935), and Müller (2006). In the context of more modern works on caecilian life history biology (Gower et al. 2008; Wilkinson et al. 2008), Seychelles caecilians are of particular interest because of their diversity and close relationship to the also reproductively

diverse Indian indotyphlids (Gower et al. 2011). The Seychelles caecilians demonstrate a variety of early life history strategies including an extended larval stage (*Praslinia cooperi*), a brief larval stage (*G. larvata* and *G. sechellensis*) and no larval stage (*H. rostratus*) (Nussbaum, 1984; San Mauro et al., 2014). Early life history strategies are unknown for *G. alternans* and *H. brevis*.

Molecular phylogenetic studies on Seychelles caecilians thus far have focused on establishing their interrelationships and their placement among caecilians globally (e.g. Frost et al., 2006; Gower et al., 2011; Loader et al., 2007; Roelants et al., 2007; San Mauro et al., 2014; Wilkinson et al., 2003, 2002; Zhang and Wake, 2009). To date only one or two individuals per nominal species has published genetic data. The results indicate that Seychelles caecilians form a monophyletic group (also indicated by studies of chromosomes: Nussbaum & Ducey 1988) that are most closely related to peninsular India caecilians (Gower et al., 2011; Hedges et al., 1993; Kamei et al., 2012; Roelants et al., 2007; San Mauro et al., 2014; Wilkinson et al., 2002). These studies have also shown that *P. cooperi* lies outside of the clade comprising the other Seychelles genera, *Grandisonia* and *Hypogeophis*, but that relationships among the five species of the latter two genera are unresolved (with the exception of the sister relationship between *G. larvata* and *G. sechellensis*) and require further investigation.

Co-distributed taxa that occur partly in sympatry and syntopy and which are closely related to each other offer an ideal opportunity to investigate factors driving evolution. The Seychelles caecilians represent a good opportunity to investigate this (see Table 1), and a combination of

morphological and molecular data would prove useful in determining if the variation observed relates to historical demography or habitat/ecological specialism.

Grandisonia alternans

Grandisonia larvata

Grandisonia sechellensis

Hypogeophis brevis

Hypogeophis rostratus

Praslinia cooperi

Fig. 6. Representatives of the six nominal species of Seychelles caecilian (photos S. Maddock, D. Gower)

Table 1 Currently known distribution data for the Seychelles amphibian taxa studied in this thesis. Distribution data is taken from (Nussbaum, 1984a; *unpub. data*).

	Southern islands					Northern islands					
	Mahé	Silhouette	Cerf	Stte Anne	Frégate	Praslin	La Digue	Félicité	Curieuse	Grand Soeur	Petite Soeur
G. alternans											
G. larvata											
G. sechellensis											
H. brevis											
H. rostratus											
P. cooperi											
T. seychellensis											

Advances in phylogenetics and phylogeography

Phylogeography has become an increasingly active area of research (Beheregaray 2008) that looks to identify patterns of geographic diversity in species (Avise 2000). Other areas of research that have seen benefits from the way that phylogeography investigates temporal changes include speciation (Avise et al. 2000; Moritz et al. 2000; Hewitt 2001; Kohn 2005), conservation biology (O'Brien 1994; Moritz & Faith 1998; Fraser & Bernatchez 2001), and taxonomy/biodiversity (Taberlet 1998; Beheregaray & Caccone 2007). To uncover the historical demographic patterns underlying evolutionary relationships between and among taxa, it is important to incorporate information from both temporal and topographic information (Donoghue & Moore 2003). Island systems offer a good opportunity for this (Emerson 2002) because of their isolation and discrete boundaries.

A lot of effort is currently being placed in to developing new tools with which to analyse molecular data to infer phylogeny and test phylogeographic hypotheses. The methods of Bayesian inference (BI) and maximum likelihood (ML) are leading the way in this (Ronquist & Huelsenbeck 2003; Drummond et al. 2006, 2012; Yang & Rannala 2010; Kubatko et al. 2009; Ence & Carstens 2011; Heled & Drummond 2010), however, new algorithms are still being trialled (Jakó *et al.* 2009).

Species tree inference has become an ever-increasing field of methodological development and many phylogenetic studies now implement some form of species tree analysis within them. These include the most complex Bayesian approaches (Liu 2008; Heled & Drummond 2010), maximum likelihood methods (Kubatko *et al.* 2009) and pseudo-likelihood

algorithms (Liu *et al.* 2010; Jewett & Rosenberg 2012). Bayesian approaches implementing the multispecies coalescent have been suggested to outperform other methods (Heled & Drummond 2010; Leaché & Rannala 2011) and therefore many recent studies have implemented these. However, few studies have systematically compared results using these newly developed algorithms (e.g. Camargo et al., 2012; Jockusch et al., 2015). Several species tree methods are used in this thesis, which provides another comparison for the utility of the individual approaches.

Estimating divergence times in order to test biogeographic hypotheses has become a common discipline. In order to increase accuracy of such analyses methods and software advancement has become prevalent (e.g. Heled & Drummond 2010). The inclusion of fossil calibrations can substantially improve accuracy. Yet the accuracy of such calibrations remains fraught with controversy and some authors prefer geological event calibrations which must also be chosen carefully (Heads 2005, 2011; Renner 2005; Emerson 2007). No-fossil calibration are often the only available option when a good fossil record is unavailable, such as in the case with hyperoliid frogs and caecilians (Evans & Sigogneau-Russell 2001).

The use of island age for dating clades has become established more recently, for which it differs from fossil calibrations by enforcing a maximum divergence age calibration rather than a minimum calibration. This however relies on the theory that island clades are the same age as the islands they inhabit, which in reality is likely to be substantially wrong in many cases (Ho & Phillips 2009). At the other end of the scale such methods could substantially underestimate cladogenic ages, which could have been around prior to island

formation (e.g. see Mendelson & Shaw 2005 and Genner et al. 2007 for likely examples).

Alternative methods of calibrating molecular clocks have subsequently been incorporated, including implementing calibrations based on changing drainage geology over time (Burridge *et al.* 2008) or abandoning calibrations altogether and instead applying a relative timescale (Loader et al. 2007). This latter option seems an ideal approach to test whether a single abiotic event caused independent divergences.

However, it seems likely that almost all of these methods will have resulted in overestimates of divergence dates (McCormack *et al.* 2011; Kubatko *et al.* 2011) prior to the implementation of the multispecies coalescent implemented through *BEAST (Heled & Drummond 2010). Prior to this analytical tool, all dating analyses were based on gene trees whether from a single locus or concatenated from multiple loci. It is a logical choice to implement species trees in such studies, considering that gene trees are not actual representations of speciation events. Gene divergences are rooted within a species tree and therefore this will inevitably result in elevated divergence estimates, assuming the absence of subsequent gene flow (Edwards & Beerli 2000).

Over the last decade sequence technology has been developing rapidly with the advance of next-generation sequencing machines. Originally, sequences generated via this method were short compared to their Sanger counterparts. However, with increasing competition between Illumina, Solexa, Roche, Pacific Biosystems, Applied Biosystems, Oxford Nanopore Technologies, Ion Torrent and Life Technologies a rapid advance in

sequencing technology has prevailed. The cost of running these systems has drastically decreased, as has processing time with the advancement of more powerful computers and increased numbers of people utilising them for data acquisition. It is now possible to sequence a human genome for ~£650 compared to ~£6,600,000 when the first next-generation sequencing machine came out in 2005 (van Dijk *et al.* 2014) and therefore the platforms have become notably more affordable.

Next-generation sequencing technologies are now used in many areas of research for example the amplification of complete mitochondrial genomes (e.g. Gillett et al., 2014), the identification of anonymous nuclear markers (e.g. Lewis et al., 2014) and microsatellites (e.g. Castoe et al., 2010), for single nucleotide polymorphism (SNP) detection (e.g. Schwartz et al., 2013), and sequence capture (e.g. Grover et al., 2012). This thesis utilises a range of methods using data generated from next-generation sequencing.

With the onset of next-generation sequencing the field of population genetics has boomed and considerably grown in statistical power. Although the traditional microsatellite approach has proved to be good across a range of taxa (e.g. Frankham 2008; Sharma et al. 2008; Barratt et al. 2011), the vast quantities of SNPs that can now be found across a genome at relatively fast speed, including within non-model organisms, provides a great resource with which to study population dynamics (Williams et al. 2010; Sharma et al. 2012; Gautier et al. 2012; Keller et al. 2012; Wheat 2012; Stölting et al. 2012). These methods allow for questions to be addressed regarding fine scale population genetic changes as well as wider phylogeographic analyses (Emerson et al. 2010).

Threats to Seychelles amphibians

As part of the Madagascan region, the islands of the Seychelles are in the top five global biodiversity hotspots (Myers et al. 2000), characterized by high levels of endemism in and conservation threats to their biota. Globally, amphibians are the most threatened class of vertebrates (Hoffmann *et al.* 2010). Amphibian populations are threatened chiefly by one or more of: habitat loss, environmental degradation, climate change, human exploitation, alien species, decreased genetic diversity, and disease (especially the potentially lethal amphibian chytrid fungus) (Allentoft & O'Brien 2010). In addition to generally high threats to amphibians globally, island populations are under greater threat of extinction than continental organisms (Frankham 2008).

Understanding diversity, distribution and origins of taxa is an important aspect of modern conservation biology. Studying the evolutionary history of Seychelles amphibians will therefore make an important contribution to the management of this unique fauna.

Thesis aims and outline

This thesis aims to document pheno- and genotypic variation within amphibians of the Seychelles, and to place these species into a phylogenetic context. Understanding intraspecific variation will help document the diversity within Seychelles biota and enable patterns and processes that lead to this variation to be identified. It also aims to improve our understanding of how amphibians evolve on isolated oceanic islands. This thesis is laid out in the following chapters:

Chapter 2 Investigates the phylogenetic relationships, evolutionary origins and intraspecific variation of the Seychelles treefrog *Tachycnemis seychellensis* using three mitochondrial and four nuclear genetic markers. Morphological intraspecific variation in *T. seychellensis* has been examined previously (Nussbaum & Wu 1995), but this is the first time that molecules have been used to assess variation within the species. The chapter also aims to estimate the time of colonisation to the Seychelles islands.

Chapter 3 Uses morphological data to discover and interpret patterns of geographic variation within and among the six nominal Seychelles caecilian species and two undescribed, newly discovered dwarf species. To fully understand evolutionary patterns and to test current taxonomy and classification it is important to investigate morphological patterns of evolution alongside molecular analyses. A total of 21 morphometric and meristic characters are used.

Chapter 4 Uses genetic data from two mitochondrial and four nuclear loci to discover and interpret diversity within and among the Seychelles caecilians and to test current taxonomy. These data complement the morphological analyses reported in Chapter 3, the combined results finding some common spatial patterns but also notable differences among species.

Chapter 5 Investigates the phylogenetic relationships among the Seychelles caecilian species using a mitogenomic approach. Multiple next-generation

sequencing technologies and methods were used to generate the data. The various methods of obtaining these novel mitogenomic data are compared and suggestions made for the best methods for obtaining mitogenomes made. Mitogenomes alone are shown to be insufficient to resolve Seychelles caecilian phylogeny.

Chapter 6 Builds on Chapter 5 by using 13 nuclear loci (a combination of coding, non-coding and anonymous nuclear loci) to infer phylogenetic relationships of the Seychelles caecilians. Seven independent methods were used to analyse the data. The results vary substantially among different methods. Seychelles caecilian phylogeny is a major challenge.

Chapter 7 Presents a summary and general discussion of the results of the thesis and their wider implications.

CHAPTER 2

EVOLUTIONARY ORIGINS AND GENETIC VARIATION OF THE SEYCHELLES TREEFROG, *TACHYCNEMIS SEYCHELLENSIS* (DUMÉRIL AND BIBRON, 1841) (AMPHIBIA: ANURA: HYPEROLIIDAE)

Abstract

The hyperoliid frog *Tachycnemis seychellensis,* the only species of its genus, is endemic to the four largest granitic islands of the Seychelles archipelago and is reliant on freshwater bodies for reproduction. Its presence in the Seychelles is thought to be the product of a transoceanic dispersal, diverging from the genus *Heterixalus*, its closest living relative (currently endemic to Madagascar), between approximately 10-35 Ma. A previous study documented substantial intraspecific morphological variation among island populations and also among populations within the largest island (Mahé). To assess intraspecific genetic variation and to infer the closest living relative(s) of *T. seychellensis,* DNA sequence data were generated for three mitochondrial and four nuclear markers. These data support a sister-group relationship between *T. seychellensis* and *Heterixalus,* with the divergence between the two occurring between approximately 11-19 Ma based on *cytb p*-distances*.* Low levels of genetic variation were found among major mitochondrial haplotype clades of *T. seychellensis* (maximum 0.7% *p*-distance concatenated mtDNA), and samples from each of the islands (except La Digue) comprised multiple mitochondrial haplotype clades. Two nuclear genes (*rag1* and *tyr*) showed no variation, and the other two (*rho*, *pomc*) lacked any notable geographic structuring, counter to patterns observed within presumably more vagile Seychelles taxa such as lizards. The low levels of

genetic variation and phylogeographic structure support an interpretation that there is a single but morphologically highly variable species of Seychelles treefrog. The contrasting genetic and morphological intraspecific variation may be attributable to relatively recent admixture during low sea-level stands, ecophenotypic plasticity, local adaptation to different environmental conditions, and/or current and previously small population sizes. Low genetic diversity but substantial morphological variation is unusual within anurans.

2.1 Introduction

Due to their isolation from potential confounding factors, remote islands have long been considered to provide important arenas for investigating evolution (Darwin 1859). Most evolutionary studies of island biotas have focused on geologically recent volcanic island groups that have never been in contact with a large, ancient mainland, for example the Galápagos and Hawaii (e.g. Darwin 1859; Gillespie 2002). In contrast, the Seychelles archipelago (1,600km east of the nearest continental landmass) is formed of both granitic and coralline islands. The current granitic Seychelles are the remaining emergent part of a continental fragment, previously part of Gondwana, that was associated with India and Madagascar when they separated from Africa during the Cretaceous. At least some of the granitic Seychelles have always had some emergent land since the break up of the Gondwanan supercontinent. Much of the continental Seychelles is currently submerged at an average depth of 55m below sea level, forming the microcontinent 'Seychellea', comprising a total area of 129,650km^2 (Davies & Francis 1964). During times of lowest stands in sea level (see Miller et al. 2005) all of the currently emergent granitic

Seychelles would have been in contact. Fluctuations in sea level likely caused many episodes of dis- and reconnection among Seychelles islands, the most recent of which were within the last 10 ka (Colonna et al. 1996; Rohling et al. 1998; Siddall et al. 2003; Camoin et al. 2004; Miller et al. 2005). These fluctuations can be expected to have had a substantial impact on the amounts and spatial patterns of genetic variation of the resident biota.

It is unsurprising that most remote islands lack an endemic amphibian fauna given that the osmotic properties of amphibian skin (e.g. Balinsky 1981; Duellman & Trueb 1986) likely reduce their dispersal capabilities over saltwater substantially (Bossuyt & Milinkovitch 2001; Darwin 1859; Myers 1953; Nussbaum 1984). The Seychelles, however, has an amphibian fauna (~12 species) comprising both frogs (Anura) and caecilians (Gymnophiona) that are restricted solely to the granitic islands (Nussbaum 1984). Except for the widespread frog *Ptychadena mascareniensis* (Duméril and Bibron, 1841) (Vences et al. 2004), all Seychelles amphibians are endemic. The endemic Seychelles frogs are confined to the four largest granitic islands of Mahé, Silhouette, Praslin and La Digue (Nussbaum & Wu 1995, 2007; Taylor et al. 2012). Except for a preliminary study of sooglossid frogs (Taylor *et al.* 2012) molecular analyses have not yet been conducted to determine patterns of genetic variation among and within populations on different islands. Molecular techniques have been applied to several other Seychelles organisms, and substantial spatial structuring and deep genetic splits have been revealed, indicating the presence of cryptic lineages within several currently recognised lizard species (Rocha et al. 2010, 2011, 2013; Valente et al., 2014), and a freshwater crab (Daniels 2011).

Hyperoliidae is a pan-African family comprising >200 species in 17 or 18 genera (AmphibiaWeb 2014; Frost 2014) of small-medium sized treefrogs with representative species also found in the Seychelles and Madagascar (Vences et al. 2003). The endemic Seychelles treefrog *Tachycnemis seychellensis*, the only species of its genus, is a sexually dimorphic, hyperoliid frog found on all four of the granitic islands of the Seychelles that support populations of frogs (Nussbaum & Wu 1995). Like all hyperoliids *T. seychellensis* is an oviparous species with an aquatic larval stage, and it is restricted to areas close to water bodies (Nussbaum 1984). The abundance and type of *T. seychellensis* habitat varies considerably across its range (Nussbaum & Wu 1995). The southern islands of Mahé and Silhouette are higher (up to 905 and 750 m elevation, respectively), wetter and dominated by moist-wet forests, whereas the northern islands of Praslin (up to 367 m) and La Digue (333 m) are much lower and drier. Praslin has multiple rivers and streams, but La Digue lacks constant water sources at higher altitudes, and instead *T. seychellensis* is restricted here to marshy areas in the low-lying plateau on the west of this small island. The sizes of the four islands vary by more than an order of magnitude, ranging from 960 ha (La Digue) to 14,480 ha (Mahé), with Silhouette (1,600 ha) and Praslin (4,040 ha) somewhat intermediate.

Using univariate and multivariate analyses, Nussbaum & Wu (1995) discovered substantial external morphological variation among five populations of *T. seychellensis* from the four islands, including in adult body size and colouration, presence or absence of tubercles on various parts of the body and limbs, presence or absence of grooved digit discs, and several

morphometric characters. Four morphometric characters, not dependant on sex, were found to vary significantly between all populations: internarial width, pes length, toe disc length, and length of metatarsal tubercle. An additional 10 male and two female characters varied significantly. Specimens from the more southerly islands of Mahé and Silhouette are morphologically the most similar to each other (Nussbaum & Wu 1995). However, within Mahé (the only island for which more than one population was sampled), two populations of *T. seychellensis* (one marsh- and one stream-associated) only 1km apart were as morphologically different from each other as they were to *T. seychellensis* on Silhouette. The populations of *T. seychellensis* on the more northerly islands (Praslin and La Digue) were morphometrically as distinct from each other as they were from the southern populations. Despite these large morphological differences, Nussbaum & Wu (1995) were impressed by (1) the fact that the four islands were likely connected as recently as 10 ka, (2) the intra-Mahé differences were as large as inter-island differences, (3) the substantial environmental differences across the four islands, and (4) the similar life history and bioacoustics of the different populations, and thus argued for the recognition of only a single species, one that has substantial and geographically structured morphological variation. Nussbaum & Wu's single-species hypothesis for *T. seychellensis* could be challenged by high genetic diversity and/or substantial phylogeographic structure.

Tachycnemis seychellensis has a complicated taxonomic history. Since Dubois (1981) the species has been included in the monotypic genus *Tachycnemis* Fitzinger, 1843 and the species name has been attributed to Duméril & Bibron (1841) with Tschudi's (1838) first use of the species name

considered unavailable. Fitzinger (1843) established *Tachycnemis* only through bibliographic reference to its single included species (as described by Tschudi, 1838) without any explanation of his biological reasons (if any) for proposing the new genus. However, it has long been considered a phenotypically rather distinct hyperoliid (Gunther 1869), and Drewes (1984) hypothesized that it is the sister group of all other extant hyperoliids. More recently, based on analysis of concatenated mitochondrial DNA (mtDNA) and nuclear DNA (nuDNA), *T. seychellensis* has been inferred to be most closely related to the endemic Madagascan genus *Heterixalus* Laurent, 1944, which has 11 currently recognized species (Frost et al. 2006; Pyron & Wiens 2011; Richards & Moore 1996; Vences et al. 2003a, 2003b; Wollenberg et al. 2007). However, although Wollenberg et al.'s (2007) main analysis of concatenated data recovered *Tachycnemis* and *Heterixalus* as sister taxa, five out of six trees inferred for the individual genes placed *Tachycnemis* within *Heterixalus*, although only *cox1* (mtDNA) and *rho* (nuDNA) did so with much support. Paraphyly of *Heterixalus* with respect to *Tachycnemis* was also found (though without strong support) by Vences et al. (2003b) in two mitochondrial gene trees. In contrast, these authors found that when three genes were concatenated, but using only two *Heterixalus* species, *Tachycnemis* and *Heterixalus* were sister taxa. Using multiple *Heterixalus* species in their analyses, Richards and Moore (1996), Frost et al. (2006), and Pyron and Wiens (2011) also recovered a *Tachycnemis-Heterixalus* sister-group relationship. The Madagascan and Seychelles hyperoliid frogs form a monophyletic group occurring within the c

ore African hyperoliid group (Richards & Moore 1996; Vences *et al.* 2003a; b; Frost *et al.* 2006; Pyron & Wiens 2011; Crottini *et al.* 2012). Based on current data it is not possible to rule out colonisation of the Seychelles before Madagascar but it is a commonly supported theory that the route of dispersal was via Madagascar and then onto the Seychelles (e.g. see Vences et al. 2003).

Where trees for individual loci are discordant in the relationship of *Tachycnemis-Heterixalus*, coalescence-based methods can be expected to yield more accurate species phylogenies than multilocus concatenation (e.g. Edwards et al. 2007; Heled & Drummond 2010; Kubatko & Degnan 2007; Maddison & Knowles 2006), but this latter approach has yet to be implemented in the case of *Tachycnemis* and *Heterixalus*. Based on a sister-group relationship with *Heterixalus* and molecular dating analyses, the presence of *T. seychellensis* in the Seychelles is considered to originate from an overseas dispersal, with *Tachycnemis* diverging from its closest African/Madagascan relative an estimated 9.79-35.34 Ma (Crottini et al. 2012), via transoceanic dispersal on a vegetation raft as suggested by Vences et al. (2003). Members of the Hyperoliidae are well documented in their ability to colonise islands across saltwater barriers via oceanic dispersal (Vences *et al.* 2003b; Bell *et al.* 2015a; b) leading to the possibility that the hyperoliid frogs are not as susceptible to desiccation as most other anurans (e.g. Balinsky 1981; Duellman & Trueb 1986). This is in contrast to the sooglossid frogs that, as with the Seychelles caecilians, have probably been resident at least since Seychellea (the Seychelles microcontinent) was last part of Gondwana (Nussbaum 1984).

Here we report phylogenetic analyses of mtDNA and nuDNA data (3,228 base pairs (bp)) to (1) test the hypothesised sister-group relationship between *Heterixalus* and *Tachycnemis* and monophyly of the former genus, and (2) assess genetic variation within *T. seychellensis* across its range and test the hypothesis that it is a single, morphologically highly variable species.

2.2 Methods

2.2.1 Taxon sampling

Tachycnemis seychellensis tissue samples (liver, heart and muscle, frozen and stored at -80°C) were obtained from 52 voucher specimens from the Seychelles islands of Mahé (15 samples), Silhouette (15 samples), Praslin (15 samples) and La Digue (7 samples) between 1988 and 1991; these correspond to four of the five populations sampled by Nussbaum & Wu (1995) (tissues of only a single Mahé population from Mare aux Cochon were available). Vouchers and tissues are deposited in the University of Michigan Museum of Zoology, USA (UMMZ).

2.2.2 Laboratory protocols

Genomic DNA was extracted from liver, heart and muscle samples from the 52 *T. seychellensis* samples using the Qiagen DNeasy™ Tissue Kit. Three mitochondrial gene fragments were sequenced for all samples: cytochrome *b* (*cytb*), cytochrome oxidase subunit 1 (*cox1*) and 16S rRNA (*16s*). Four nuclear loci were also sequenced: rhodopsin exon 1 (*rho*), recombination activating gene 1 (*rag1*), tyronsinase precursor (*tyr*) and pro-opiomelanocortin (*pomc*).

The *rag1* and *tyr* sequences showed no variation within *T. seychellensis,* and thus only a subset of individuals from each locality were included in the analyses of the relationships between *Tachycnemis* and *Heterixalus*.

Primer information is given in Table 1. Sequences were amplified using the polymerase chain reaction (PCR) with a total reaction volume of 15µl: 1.5µl of Bioline Buffer, 0.75µl of MgCl2, 0.15µl of dNTPs, 0.15µl of Taq, 06µl of both the forward and reverse primers, 0.6µl of template DNA, and 10.65µl ddH_2O. Cycling conditions were: denature at 94°C for 60s; followed by 35 (*16s*, *cytb*,) or 40 (*cox1, tyr, pomc, rag1*) cycles of denaturing at 94°C for 30s, annealing at 48°C (*cox1*), 50°C (*16s*), 52°C (*cytb*), 56°C (*rag1*), 60°C (*rho*), or 62°C (*tyr, pomc*) for 30s, and extending at 72°C for 30s; and a final extending step of 72°C for 5min.

Table 1. Primers used in this chapter for PCR and sequencing.

Gene Fragment	Primer	Sequence (5' – 3')
16s	16SA-L [a]	CGCCTGTTTATCAAAAACAT
	16SB-H [a]	CCGGTCTGAACTCAGATCACGT
cox1	Amp-P3 F [b]	CAATACCAAACCCCCTTRTTYGTWTGATC
	Amp-P3 R [b]	GCTTCTCARATAATAAATATYAT
cytb	L14841 [c]	CTCCCAGCCCCATCCAACATCTCAGCATGATGAAACTTCG
	CB3H [d]	GGCAAATAGGAAGTATCATTCTG
pomc	POMC-1 [e]	GAATGTATYAAAGMMTGCAAGATGGWCCT
	POMC-2 [e]	TAYTGRCCCTTYTTGTGGGCRTT
tyr	Tyr1C [f]	GGCAGAGGAWCRTGCCAAGATGT
	Tyr1G [f]	TGCTGGGCRTCTCTCCARTCCCA
rho	Rhod1A	ACCATGAACGGAACAGAAGGYCC
	Rhod1D	GTAGCGAAGAARCCTTCAAMGTA
rag1	Amp-RAG1 F [b]	AGCTGCAGYCARTACCAYAARATGTA
	Amp-RAG1 R1 [b]	AACTCAGCTGCATTKCCAATRTCACA

[a] Palumbi et al. (1991)
[b] San Mauro et al. (2004)
[c] Kocher et al. (1989)
[d] Moritz et al. (1992)
[e] Wiens et al. (2005)
[f] Bossuyt and Milinkovitch (2000)

2.2.3 Genetic variation within Tachycnemis seychellensis

Sequences were proof-read using Sequencher v.4.8 and initially aligned using ClustalX v.2.0 (Larkin *et al.* 2007) using default settings before being checked by eye. All genes except the non-protein-coding *16s* were checked for pseudogenes and insertions by searching for stop codons and indels (e.g. Zhang & Hewitt 1996) in MEGA5 (Tamura *et al.* 2011). The program DAMBE (Xia & Xie 2001) was used to test for saturation using the test of Xia et al. (2003) across the different codon positions and the combined dataset.

To infer the phylogenetic relationships within *T. seychellensis* for the mitochondrial locus, we used Bayesian inference (BI) implemented in BEAST v.1.7.4 (Drummond *et al.* 2012). No outgroup taxa were used because BEAST estimates the position of the root in the tree assuming a molecular clock (Heled & Drummond 2010). Input XML files were generated for BEAST analyses using BEAUti v.1.7.4. We selected best partitioning strategies and BEAST-compatible substitution models using PartionFinder (Lanfear *et al.* 2012).

The coalescent tree prior with exponential growth was used in BEAST based on the assumption that, after an initial colonisation, *T. seychellensis* likely expanded its range. Following the results of initial runs, uncorrelated relaxed clocks were rejected for all partitions and a strict clock implemented because constant rates could not be rejected. Two MCMC chains were run for 1×10^8 generations for each partitioning strategy, with trees sampled every 10,000 generations to ensure convergence; this gave a total of 10,000 output trees per run. Convergence was checked by manual observation of the trace plots and ESS scores using Tracer v.1.5 (Rambaut & Drummond 2009). All

BEAST analyses were performed using the CIPRES Science Gateway v.3.1 (Miller et al. 2010).

To infer allelic phases from polymorphic sites in the nuDNA, the program PHASE 2.1 (Stephens et al. 2001; Stephens & Scheet 2005) was used, with input files created using seqPHASE (Flot 2010). Haplotype networks under the median-joining algorithm (Bandelt *et al.* 1999) were produced to display intraspecific variation for *T. seychellensis* for the *pomc* and *rho* loci using the program NETWORK v.4.611 (fluxus-engineering.com).

Tajima's *D* (Tajima 1989) and Fu's *Fs* (Fu 1997) neutrality tests were used to investigate historical demographic properties in each island population of *T. seychellensis*. Negative values indicate a recent population expansion, values close to zero indicate a stable population, and any values considerably over zero indicate a recent population decrease. Both *D* and *Fs* were calculated using Arlequin v.3.5.1.3 (Excoffier & Lischer 2010). Input files for Arlequin were created using PGDSpider v.2.0.3.0 (Lischer & Excoffier 2012).

Table 2 Partitioning schemes and substitution models for the *Tachycnemis seychellensis* intraspecific mtDNA dataset. Numbers in parentheses refer to codon position.

	Partition scheme	**Substitution models**
AIC / AICc	*16s*	TrN
	cytb (1), *cox1* (1)	HKY
	cytb (2), *cox1* (2)	HKY
	cytb (3)	TrN
	cox1 (3)	TrN + G
BIC	*16s, cytb* (1), *cox1* (1)	HKY
	cytb (2), *cox1* (2)	HKY
	cytb (3), *cox1* (3)	TrN + G

2.2.4 Testing monophyly of Heterixalus

The multispecies coalescent method as implemented in *BEAST (Heled & Drummond 2010) was used to infer the species trees for *Tachycnemis* and *Heterixalus* spp., treating mtDNA (*cytb, cox1* and *16s*), *tyr*, *rag1* and *rho* as four separately evolving loci. Sequence data for *Heterixalus* spp. were previously published (Wollenberg *et al.* 2007) and obtained from GenBank, and those for *Tachycnemis* were newly generated. It is recommended to include a minimum of two specimens per species for *BEAST analyses so that there is a coalescent event with which to estimate population size (Heled & Drummond 2010), but this was not possible for all species of *Heterixalus* because of inadequate specimen and/or character sampling in GenBank. For this reason *H. alboguttatus, H. boettgeri* and *H. carbonei* were excluded from these analyses. The remaining taxa nonetheless included representatives of all five *Heterixalus* species groups identified by Wollenberg et al. (2007).

Many studies using multilocus datasets do not partition by codon position, but we ran two sets of analyses in order to test for discrepancies between this ad hoc method and the optimal partitioning strategy identified by PartitionFinder (Table 3). Due to over parameterization, convergence was not reached in the identified optimal partitioning scheme in further analyses and therefore only the results of the locus partitioned analysis is used.

Preliminary analyses of the locus-partitioned dataset suggested that strict clocks be implemented for the mtDNA and *rhod* partitions and an uncorrelated lognormal relaxed clock for the *tyr* and *rag1*. Rates for molecular clocks were initially set at default (1.0) for all partitions and estimated relative to the mtDNA partition. Two MCMC chains were run for 2 x 10^8 generations,

with trees sampled every 10,000 generations, to ensure convergence was reached the first 5% were discarded as burn-in, although convergence was reached prior to this cut-off. The species-tree Yule-process prior was used with the piecewise linear and constant-root population-size model. Convergence of all parameters was verified using Tracer v.1.5 (Rambaut & Drummond 2009).

Table 3 Best-fit substitution models for partitions for the multispecies coalescent analysis.

	Partition	**Substitution models**
Locus partitions	mtDNA	GTR + G
	rag1	TrN + I
	rho	GTR + G
	tyr	SYM + I + G

2.3 Results

2.3.1 Monophyly of Heterixalus?

We aligned 1,500 bp of mtDNA (consisting of 424 variable sites (v.s.), of which 391 were parsimony informative (p.s.)), 760 bp of *rag1* (62 v.s., 48 p.s.), 357 bp of *rho* (30 v.s., 15 p.s.), and 611 bp of *tyr* (56 v.s., 45 p.s.) giving a total sequence length of 3,228 bp. Partitioning schemes and nucleotide models used in analyses are presented in Table 3.

Partitioning the *BEAST dataset by linked loci provided evidence for *T. seychellensis* being the sister taxon to a monophyletic *Heterixalus*; a sister-group relationship between *H. madagascariensis* and *H. punctatus*; for *H. andrakata* being the sister taxon to *H. tricolor* + *H. variabilis*; and for *H. betsileo* being the sister taxon to the *andrakata*-*tricolor*-*variabilis* clade (Fig. 1).

The relationships of the remaining two species (*H. rutenbergi* and *H. luteostriatus*) are unresolved (Fig. 1).

Among *Heterixalus* species and clades, mean *p*-distances for *cytb* range from 3 to 19.5%, with the *p*-distance between *Tachycnemis* and *Heterixalus* being 21% (Fig. 1). Given an approximate rate of 0.6-1% per million years for *cytb* in amphibians (see Elmer et al., 2007), this marker indicates that *T. seychellensis* diverged from its closest sampled relative in the region of 11.5-19.2 Ma.

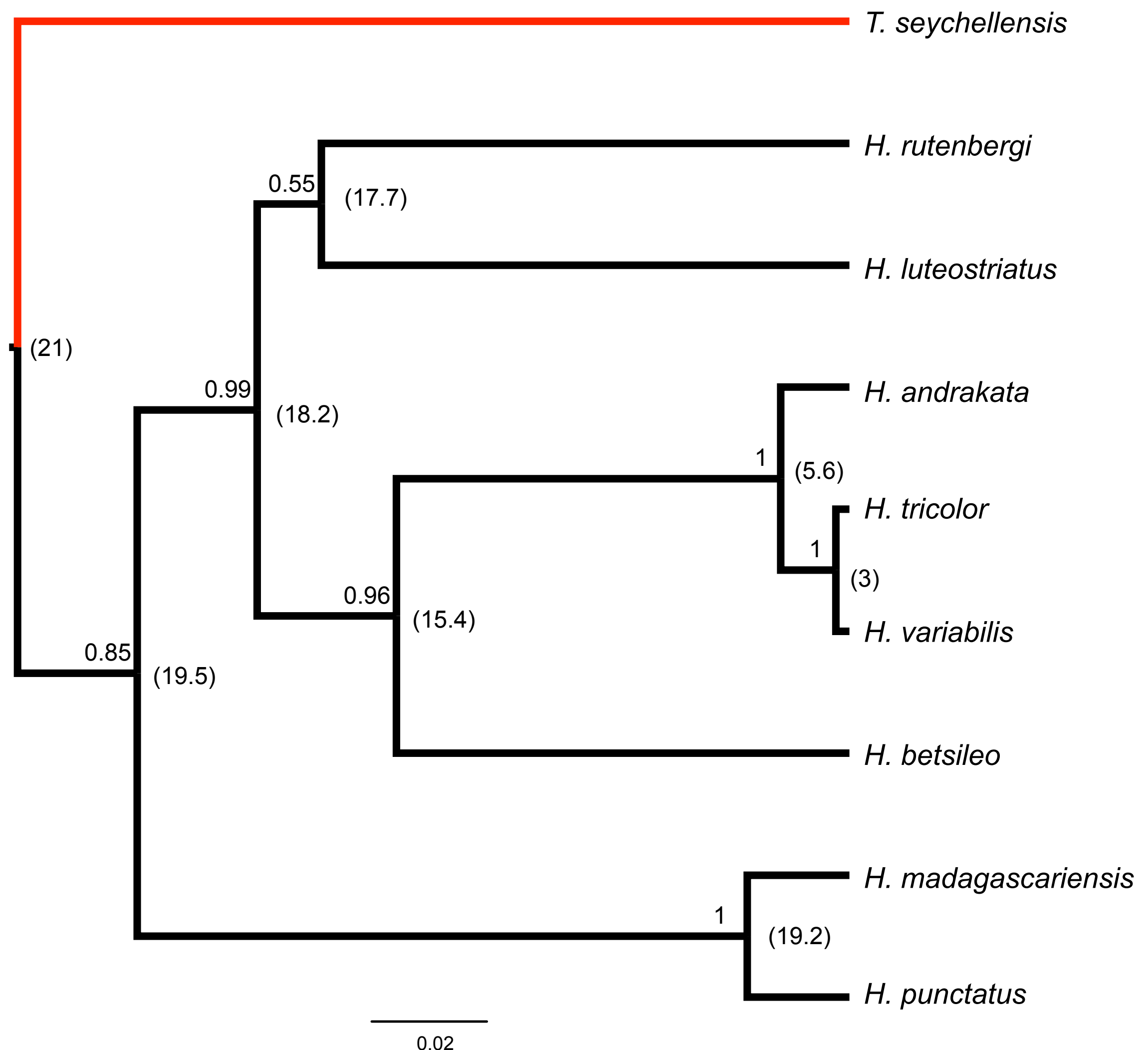

Fig. 1. Bayesian species tree of the relationships between *Tachycnemis* and *Heterixalus* inferred using the multispecies coalescent in *BEAST. Numbers on branches are Bayesian posterior probabilities. The red branch indicates the placement of *T. seychellensis* whereas those of *Heterixalus* spp. are black. Numbers in parentheses at nodes are mean *p*-distances for *cytb* between two lineages.

2.3.2 Genetic variation within Tachycnemis seychellensis

We aligned three mitochondrial genes for the 52 specimens: *16s* consisted of 599 bp with three variable sites (v.s.), all of which were parsimony-informative (p.s.); *cytb* 763 bp (32 v.s., 30 p.s.); and *cox1* 786 bp (16 v.s., 13 p.s.). The dataset was almost complete, with very little missing sequence data across all genes and no genes missing for any individual. No saturation was detected. The *rag1* and *tyr* data were constant in 20 and 19 samples sampled across all populations, respectively. The *pomc* data consisted of 629 bp (7 v.s., 6 p.s.) for 50 specimens; and *rho* 337 bp (2 v.s., 2 p.s.) for 25 specimens. The sample size for *pomc* and *rho* was reduced because of a shorter amplified sequence length of some samples and because of a lack of confidence in the accuracy of the PHASE calling of the small number of variable sites.

The best partitioning strategies and models as determined by PartitionFinder for the mtDNA analyses were the same under AIC and AICc but different under BIC (Table 2). Thus, two BEAST analyses were run under these alternatives, and the resulting tree topologies were identical and support values nearly so (Fig. 2).

The mtDNA has a maximum *p*-distance of 1.5% between any of the seven haplotypes, and no simple geographic structure is observed in the mtDNA tree (Fig. 3), with samples from all islands except La Digue comprising two haplotype clades that are not sister groups, although not all relationships are well supported. The mean *p*-distance for *cytb* among the main mtDNA haplotype clades ranges from 0.4 to 1.5% (Fig. 2). Given an approximate rate of 0.6-1% per million years for *cytb* in amphibians (see Elmer et al., 2007), this

marker suggests that extant mtDNA haplotype lineages of *T. seychellensis* began diverging approximately 0.75-1.25 Ma.

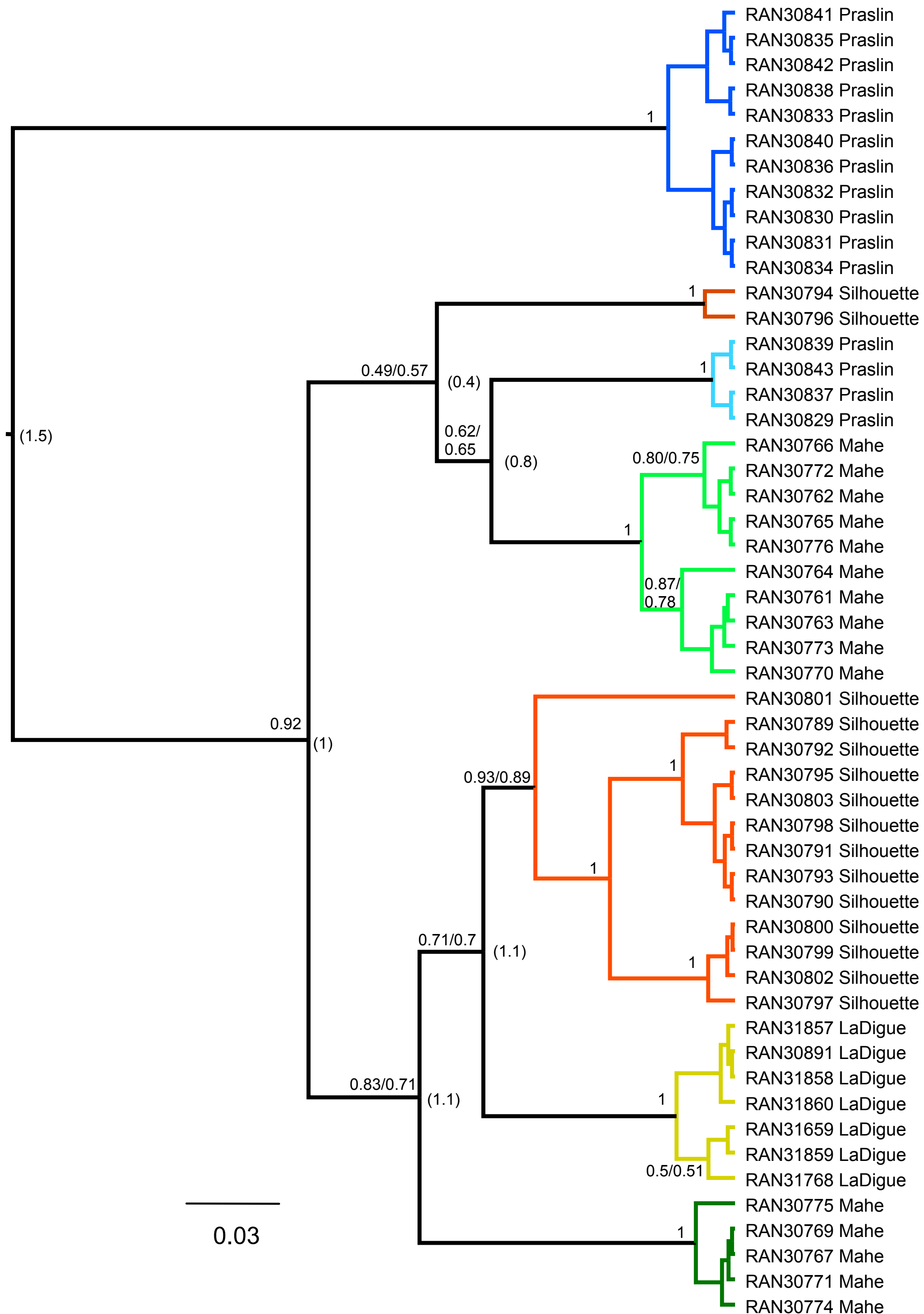

Fig. 2. Bayesian inference tree for *Tachycnemis seychellensis*_using three mtDNA gene fragments (*16s, cytb, cox1*) analysed with the BEAST software package. Numbers on branches are Bayesian posterior probabilities under AIC / BIC; when a single number is used both AIC and BIC schemes produced the same BPPs. Clade colours refer to those used in Fig. 3. Numbers in parentheses at nodes are mean *p*-distances for *cytb* between two lineages.

The two variable nuclear genes yielded networks with a general lack of geographic structure (Fig. 3). For *pomc* (Fig. 3a) there is a small amount of population structuring, with endemic haplotypes shared by multiple individuals within Praslin and Mahé. For *rho* (Fig. 3b) each of the four haplotypes are found on all islands except La Digue (two haplotypes).

Fu's *Fs* results indicate recent expansions for all of the island populations with maximal significance (Table 4). Tajima's *D* values suggest an opposite trend, with positive values indicating either a population size decrease or balancing selection, but Tajima's *D* results are not significant for any island (Table 4).

Table 4 Population genetic statistics for Fu's *Fs* and Tajima's *D* for mtDNA data for 52 *Tachycnemis seychellensis*.

Island	***N***	***Fs***	***p* - values**	**Tajima's D**	***p* - values**
Mahé	15	-8.99022	0.00000	1.59096	0.96300
Silhouette	15	-11.24523	0.00000	0.24311	0.62500
Praslin	15	-10.71708	0.00000	1.52485	0.95600
La Digue	7	-9.21700	0.00000	0.20619	0.65400

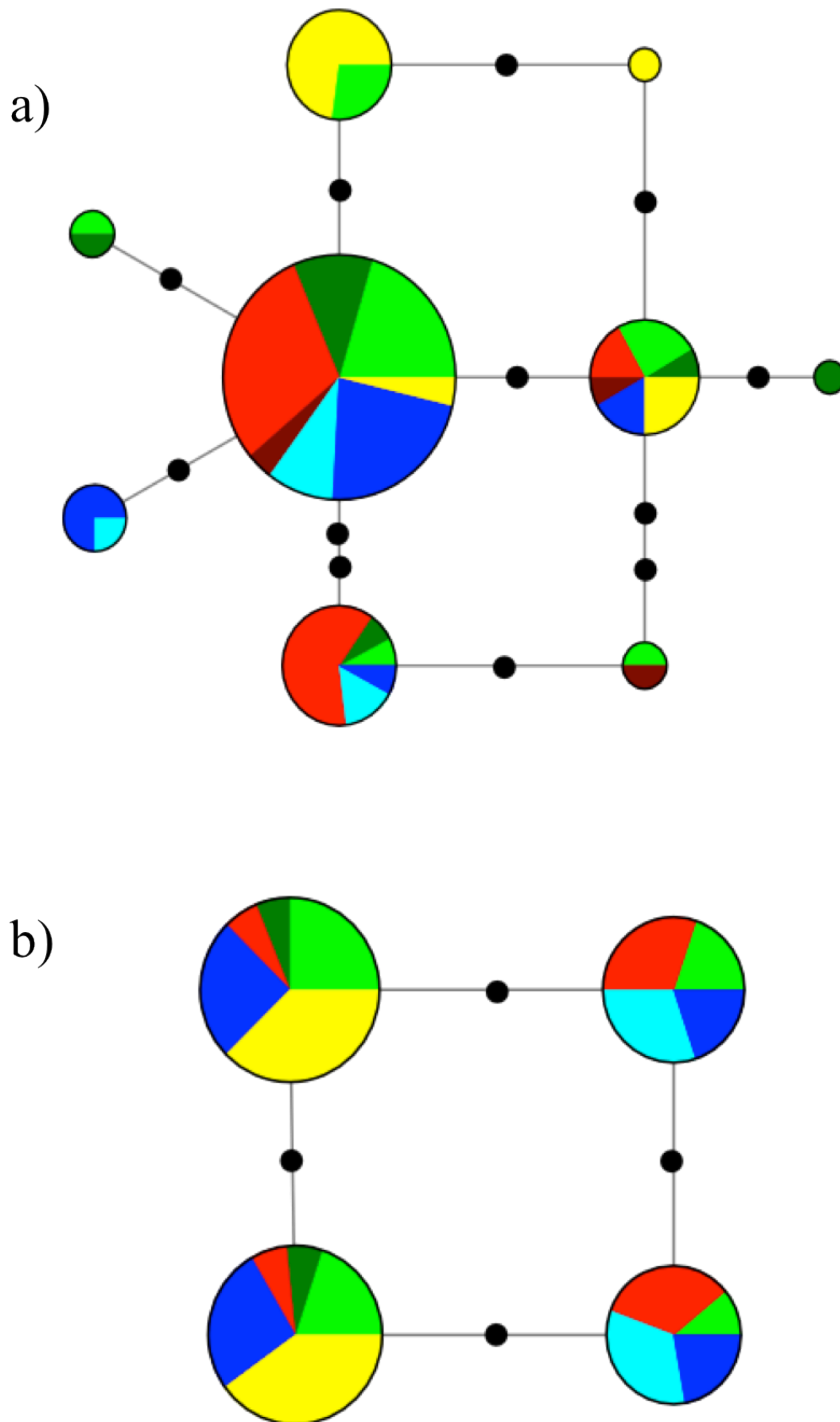

Fig. 3. Median-joining haplotype networks for two nuDNA genes for *Tachycnemis seychellensis* determined using NETWORK: a) *pomc*; b) *rho*. Segment colours refer to clades in the mtDNA phylogenetic tree (Fig. 2). Black circles on connecting branches indicate the number of mutational steps between haplotypes.

2.4 Discussion

2.4.1 Monophyly of Heterixalus?

It has been estimated that *Tachycnemis seychellensis* diverged from its closest living relative in Madagascar 9.79-35.34 Ma (Crottini *et al.* 2012), which implies transoceanic dispersal to the Seychelles given that this microcontinent split from Madagascar approximately 84 Ma (Plummer & Belle 1995; Ali & Aitchison 2008) and India by 64 Ma (Chatterjee et al., 2013; Collier et al., 2008; Davies, 1968; Dickin et al., 1986; Mart, 1988; McElhinny, 1970; McKenzie and Sclater, 1971; Norton & Sclater 1979). Transoceanic dispersal remains a rarely documented phenomenon in amphibians, but see Hedges et al. (1992); Measey et al. (2007); Vences et al. (2003, 2004).

Our analyses using the multispecies coalescent support previous studies based on concatenated multilocus DNA sequence data (e.g. Pyron & Wiens 2011; Vences et al. 2003a, 2003b; Wollenberg et al. 2007) that have hypothesized *T. seychellensis* to be the sister taxon to *Heterixalus*. Translation of *cytb p*-distances to divergence times among lineages produces estimates that fall within Crottini et al.'s (2012) estimate of 9.79-35.34 Ma for the divergence between *T. seychellensis* and its closest living relative. The *cytb p*-distances between *Tachycnemis* and *Heterixalus* spp. (Fig. 1) are clearly more in agreement with overseas dispersal than Seychelles-Africa or Seychelles-Madagascar Cretaceous vicariance as an explanation for the origin of *Tachycnemis* in the Seychelles.

2.4.2 Genetic variation within Tachycnemis seychellensis

The results of our genetic analyses are consistent with Nussbaum & Wu's (1995) interpretation that the Seychelles treefrog represents a single species. The low levels of genetic diversity within *T. seychellensis* and lack of notable phylogeographic structure can be explained by a rapid range expansion (supported by results for Fu's *Fs*) and/or by multiple admixture events possibly during eustatic sea-level fluctuations. The latter is plausible given that all island populations, apart from La Digue, have multiple mtDNA haplotype clades and that nuDNA haplotypes show no clear geographic structuring. The relatively low levels of genetic variation within the Seychelles treefrog are comparable with several other Seychelles taxa such as *Drosophila* flies (Legrand *et al.* 2009) and freshwater turtles (Silva et al., 2010), although the turtles are probably a recent human introduction (Fritz et al., 2013). Conversely, studies of other taxa including lizards (Rocha et al. 2010a, 2010b, 2011, 2013; Valente et al., 2014), a freshwater crab (Daniels 2011), and a sooglossid frog (Taylor *et al.* 2012) have revealed much higher levels of inter-island genetic variation. It remains to be fully assessed whether differences in patterns of genetic variation among Seychelles organisms can be explained by ecology (and dispersal ability) and/or duration of residency. The presence of low genetic diversity and little phylogeographic structure but high morphological variation as is observed in *T. seychellensis* is unusual in (at least adult) anurans and it is difficult to find any examples in the literature (though see e.g., Gvoždík et al., 2008, 2010).

The combination of low levels of genetic diversity (and little phylogeographic structure) within *T. seychellensis* yet substantial

morphological variation is perhaps best explained by rapid local adaptation to different environmental settings, ecophenotypic plasticity, or from previous genetic bottlenecks and/or continuing small population sizes (see also Nussbaum & Wu 1995). The latter explanation seems unlikely on the islands of Mahé and Praslin where *T. seychellensis* is abundant in the sampled populations (STM, RAN, DJG *pers. obs.*). These explanations could be tested using population-genetic analyses of data from more rapidly evolving nuclear markers.

Genetic (*cytb p*-) distances between populations of *T. seychellensis* on different islands (see Fig. 2) provide no evidence for admixture between the islands after 200-333 ka. This might suggest that during the most recent sea-level fluctuations (~10 ka), where all islands would have been connected (Colonna et al. 1996; Rohling et al. 1998; Siddall et al. 2003; Camoin et al. 2004; Miller et al. 2005), little migration occurred or, if migration did occur, mitochondrial haplotypes did not become fixed.

2.4.3 Conclusions

We find some support for the sister-group relationship between *Tachycnemis seychellensis* and a monophyletic *Heterixalus*. There is little genetic variation within *T. seychellensis,* even among populations on the four different islands within its range, and the variation is not strongly spatially structured. This is consistent with Nussbaum & Wu's (1995) interpretation that there is a single species of Seychelles treefrog. The patterns of genetic variation that we have discovered do not allow us to reject Nussbaum & Wu's (1995) proposal that substantial morphological variation within *T. seychellensis* is the result of local

ecological adaptation and/or small population sizes now and/or in the past, though ecophenotypic plasticity might also be considered.

CHAPTER 3

INTRASPECIFIC MORPHOLOGICAL VARIATION IN THE SEYCHELLES CAECILIAN AMPHIBIANS (AMPHIBIA: GYMNOPHIONA: INDOTYPHLIDAE)

Abstract

Patterns of geographic variation in the fauna and flora of the Seychelles archipelago are generally poorly studied and most published works investigating intraspecific across-island variation have thus far focussed on molecular datasets. This chapter examines intraspecific morphological variation and sexual dimorphism in the six nominal Seychelles caecilian species and two newly discovered undescribed species. Data are generated for 21 characters across 580 specimens. Four (*G. alternans, G. larvata, H. brevis* and *H. rostratus*) out of the six species show geographically structured intraspecific morphological variation. For all four of these species, morphological variation is partitioned primarily into northern- and southern-island groups. Within the southern- island group, there is some clustering of the islands of Silhouette vs. Mahé (*G. larvata*) and Silhouette vs. Mahé+Frégate (*G. alternans*). It is likely that the patterns observed are due in part to repeated episodes of eustatic sea-level fluctuations, though local ecological adaptation cannot be ruled out. Additionally, sexual dimorphism is identified in the closely related *Grandisonia larvata* and *G. sechellensis,* and the most widespread species, *Hypogeophis rostratus*.

3.1 Introduction

Animal radiations often display high levels of morphological variation in response to differing selection regimes across divergent ecological niches. Adaptive radiations have been studied extensively in model organisms such as cichlid fishes (e.g. Rüber *et al.* 1999; Seehausen 2006; Keller *et al.* 2012; Brawand *et al.* 2014), *Heliconius* butterflies (e.g. The Heliconius Genome Consortium 2012; Nadeau *et al.* 2013) and *Anolis* lizards (e.g. Wegener *et al.* 2014; Thorpe *et al.* 2015; Klaczko *et al.* 2015). However, very few studies have attempted to look at small and/or relatively ancient radiations of closely related but morphologically distinct amphibians.

Only recently have lower-level evolutionary studies on caecilian amphibians (order Gymnophiona) been conducted beyond the description of new species (Nussbaum & Pfrender 1998; Gower *et al.* 2007; Stoelting *et al.* 2014; Wang *et al.* 2015). One reason for this lack of study is likely due to the dedicated effort required to collect viable samples to effectively and rigorously investigate variation within species, considering that most caecilians are soil dwelling and rarely encountered opportunistically.

The Seychelles archipelago is part of the biodiversity hotspot that also includes Madagascar, Mauritius, Reunion and the Comoros (Myers *et al.* 2000). The Seychelles is rich in endemic amphibians, especially for an isolated island group. The amphibian fauna includes a small but morphologically diverse radiation of caecilians (Nussbaum 1984a). This caecilian radiation consists of six nominal species, all of which can be found together within particular single localities (pers. obs.; R.A. Nussbaum *unpub. data*), suggesting that some ecological differences likely occur among the

species. The Seychelles caecilian species are also all distributed across multiple islands, with the exception of one (*Hypogeophis brevis*) (Nussbaum 1984; *pers. obs.*) restricted to the largest island of Mahé. The Seychelles caecilians have been isolated from their closest extant relatives in India (Gower *et al.* 2011; San Mauro *et al.* 2014) since the Seychelles and India separated ~ 65 MYA (Gunnell *et al.* 2003; Collier *et al.* 2008; Armitage *et al.* 2011).

Within the Seychelles few organisms have been examined morphologically to investigate intraspecific variation within and among islands. Work on morphological differences in the Seychelles treefrog *Tachycnemis seychellensis* discovered strong spatial structuring among the five populations that were sampled, including between two populations occurring on the island of Mahé (Nussbaum & Wu 1995), however, genetic variation is largely not spatially structured (Maddock *et al.* 2014; Chapter 2). The conflict between the results of the morphological assessment of *T. seychellensis* and that of molecular analyses support a need for a thorough assessment of both morphology and molecular data to fully understand the diversity of Seychelles organisms and its evolutionary cause(s). Other studies utilising only molecular techniques to investigate intraspecific variation in Seychelles organisms have found differing geographical patterns: a northern- vs. southern- island group (lizards (*Phelsuma* and *Urocotyledon* geckoes, and *Mabuya* and *Pamelaescincus* skinks): Radtkey 1996; Rocha *et al.* 2010a, 2011, 2013a; Valente *et al.* 2014; *Drosophila* flies: Legrand *et al.* 2011); northern-islands, Silhouette and Mahé(+Frégate) groups (freshwater crab: Daniels 2011; sooglossid frog: Taylor *et al.* 2012); two southern-island populations (Mahé vs.

Silhouette) (sooglossid frog: Van Der Meijden *et al.* 2007); and no geographic structuring (*Drosophila* flies: Legrand *et al.* 2009; freshwater turtles: Silva *et al.* 2010).

Because the six nominal Seychelles caecilian species occur in sympatry at some localities on the largest island of Mahé it is theoretically possible that diversification of the radiation could have been driven (at least in part) by sexual selection rather than ecological adaptation or geographical isolation. Sexual selection has been identified as a causal factor in the speciation of many organisms (e.g. Price 1998; Panhuis *et al.* 2001; Pauers & Mckinnon 2012; Wagner *et al.* 2012; Klaczko *et al.* 2015) yet few studies have studied sexual dimorphism within caecilians (see Kupfer 2009). Those studies that have found sexual dimorphism in caecilians have usually found that males have greater head lengths and widths, as well as smaller body sizes (see Kupfer 2009). Only one Seychelles caecilian, *Hypogeophis rostratus*, has been investigated for sexual dimorphism, showing that males have larger heads than females (Nussbaum & Pfrender 1998).

The study reported in this chapter generates and analyses data for 21 morphological characters from 580 specimens of Seychelles caecilians from across most of their range, representing all nominal species and two undescribed dwarf species (discussed throughout the text as *H.* cf. *brevis* CR and *H*. cf. *brevis* Praslin or together with *H. brevis sensu stricto* as the *H. brevis* group), from across most of their range. The aim of the chapter is to test for sexual dimorphism within each species and to identify major patterns in geographic variation in morphology. The monophyly and broadly similar but different ecology of Seychelles caecilian species (soil dwelling carnivores

preferring moist forest habitats) suggests a null hypothesis that all of the Seychelles caecilians will exhibit the same pattern of morphological variation over the same geographical distributions. Support for this comes from the common patterns observed among Seychelles lizards (Radtkey 1996; Rocha *et al.* 2010a, 2011, 2013a; Valente *et al.* 2014).

3.2 Methods

Northern islands of the granitic Seychelles are considered to be those occurring north of 4°24'17.91"S latitude (Praslin, La Digue, Félicité, Curieuse). Those islands to the south, with the exception of Frégate, are considered to be southern islands. The easternmost island of Frégate (55°56'28.16"E longitude) is referred to as an intermediate island or the easternmost island.

Morphological data were gathered from alcohol preserved museum specimens from the University of Michigan Museum of Zoology (UMMZ), the Natural History Museum, London (BMNH) or collected during fieldwork between 2013 and 2015. Specimens of approximately average sizes were selected, where possible, for each species based on approximate examination by eye. These selected specimens excluded juveniles because during development certain characteristics, most notably the tentacle which starts at the eye and migrates towards the naris during development, would bias results. During recent fieldwork two superficially distinct populations of *H. brevis*-like caecilians were discovered. These were found beyond the previously known distribution of *H. brevis*, and their morphological variation lay substantially outside variation of the species and that they are potentially distinct, undescribed species (treated as *H.* cf. *brevis* CR and *H.* cf. *brevis*

Praslin – see section 3.1 for further details). Morphological data was gathered for 580 specimens across the six nominal species (*G. alternans n* = 101 (34 males, 58 females, nine unknown sex), *G. larvata n* = 49 (22 males, 27 females), *G. sechellensis n* = 41 (18 males, 23 females), *H. brevis* = 39 (11 males, 19 females, nine unknown sex), *H. rostratus* = 315 (169 males, 146 females), *P. cooperi* = 15 (10 males, five females)) and *H.* cf. *brevis* CR (*n* = 5 males) and *H.* cf. *brevis* Praslin (*n* = 15 (six males, six females, three unknown sex). The dataset covered all islands that each species is distributed across, with the exception of the northern islands of Grand Soeur and Petite Soeur and the southern island of Cerf for *H. rostratus* (see Fig. 1 for sampling localities). All measured lengths are in mm and were recorded using a Helior™ dial vernier caliper, apart from total length and body circumference which were measured using a ruler. Body circumference was accomplished by wrapping string around the specimen at mid-body and measuring against a ruler. The sex of each specimen was determined by physical examination of gonads examined through ventral incisions. The morphometric measurements recorded were: head length (HL), from tip of snout to the first collar groove directly behind the corner of the mouth; lower jaw length (BJL), from tip of bottom jaw directly behind the corner of the mouth; head width (HW), at posterior corner of mouth; eye-eye distance (IO), the shortest distance between the eyes; internarial distance (IN), the shortest distance between the nares; eye-naris (EN), the shortest distance between the eye and naris; eye-tentacle (ET), the shortest distance between the eye and the tentacle; tentacle-naris (TN), the shortest distance between the tentacle and the naris; tentacle-tentacle (TT), the shortest distance between the tentacles; length of

the first collar (C1), measured directly behind the corner of the mouth; length of the second collar (C2), measured directly behind the corner of the mouth (see Fig 2). The meristic counts were: number of transverse grooves on the dorsal surface of the first collar (C1-TG); number of transverse grooves of the ventral surface of the first collar (C1-VG); number of tertiary grooves on the dorsal surface of the second collar (C2-TG); distance behind vent (tail length), measured from tip of tail to centre of vent; number of primary annular grooves (PAG); number of PAGs anterior to first secondary annular groove (SAG); number of PAGs anterior to first dorsally complete SAG followed by consistently dorsally-complete SAGs (dSAG); positions of dorsally complete SAGs, counted in PAGs, if not followed by consistently dorsally complete SAGs (fcSAGs); number of PAGs anterior to first ventrally-complete SAG (vSAG); and number of annular grooves interrupted by vent disc (VAG), to tail tip. The number of scale rows and number of PAG containing scales was also recorded for a number of animals initially, however, due to fear of damaging specimens and time constraints these characters were subsequently rejected.

All analyses were carried out using PAST v.3.05 (Hammer *et al.* 2001). Measurement data were first transformed relative to body length using the 'allometric vs. standard' method (Elliot *et al.* 1995), which estimates allometric coefficients with respect to a standard/reference measurement. Multivariate analysis of variance (MANOVA) scores were first calculated using Wilks' lambda to test whether sexual dimorphism of head characteristics (based on previous studies results of caecilian sexual dimorphism (see Kupfer 2009)) is exhibited by any of the Seychelles caecilians and whether island populations are significantly different from each other. If the MANOVA results reported

sexual dimorphism within a species subsequent analyses were carried out separately. Each species was subjected to principal component analyses (PCAs) and principal coordinate analyses (PCoAs). PCAs were used for analyses of only morphometric data, whereas PCoAs were used when morphometric and meristic data were used together. If sexual dimorphism was suggested as occurring within the species in the MANOVA results then PCA analyses of head measurements between sexes were conducted (based on the findings of sexually dimorphic characters published for caecilians previously (see Kupfer 2009)). In all of the analyses each species was analysed separately except for *H. brevis* and the two *H.* cf. *brevis* population (CR and Praslin) that were analysed together.

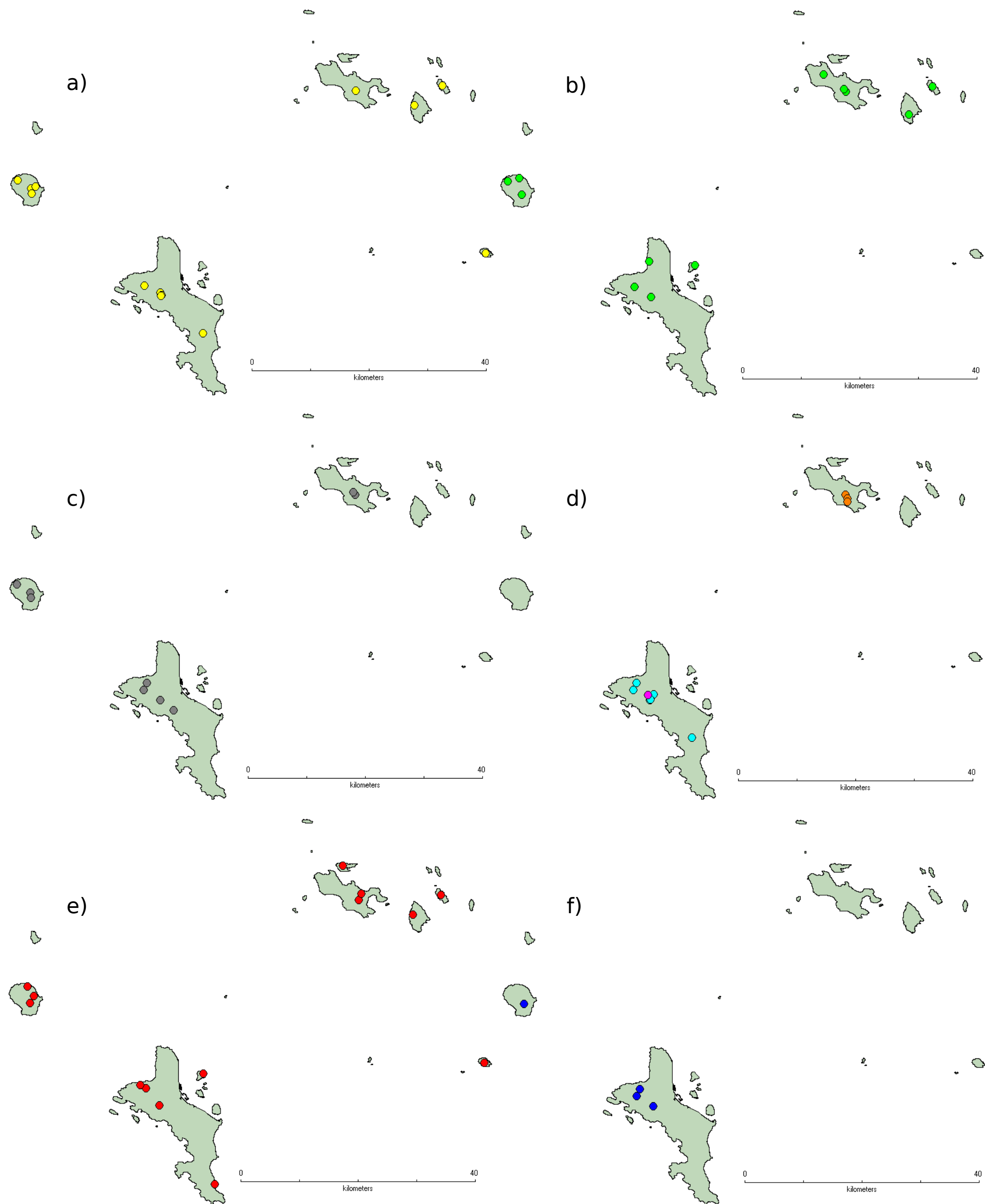

Fig. 1. Maps of the granitic Seychelles showing morphological sampling localities of each species of caecilian studied in this chapter: a) *Grandisonia alternans*, b) *G. larvata*, c) *G. sechellensis*, d) *Hypogeophis brevis* group, e) *H. rostratus*, f) *Praslinia cooperi*. Points used on each map are the same colours used for interspecific analyses in Chapter 4.

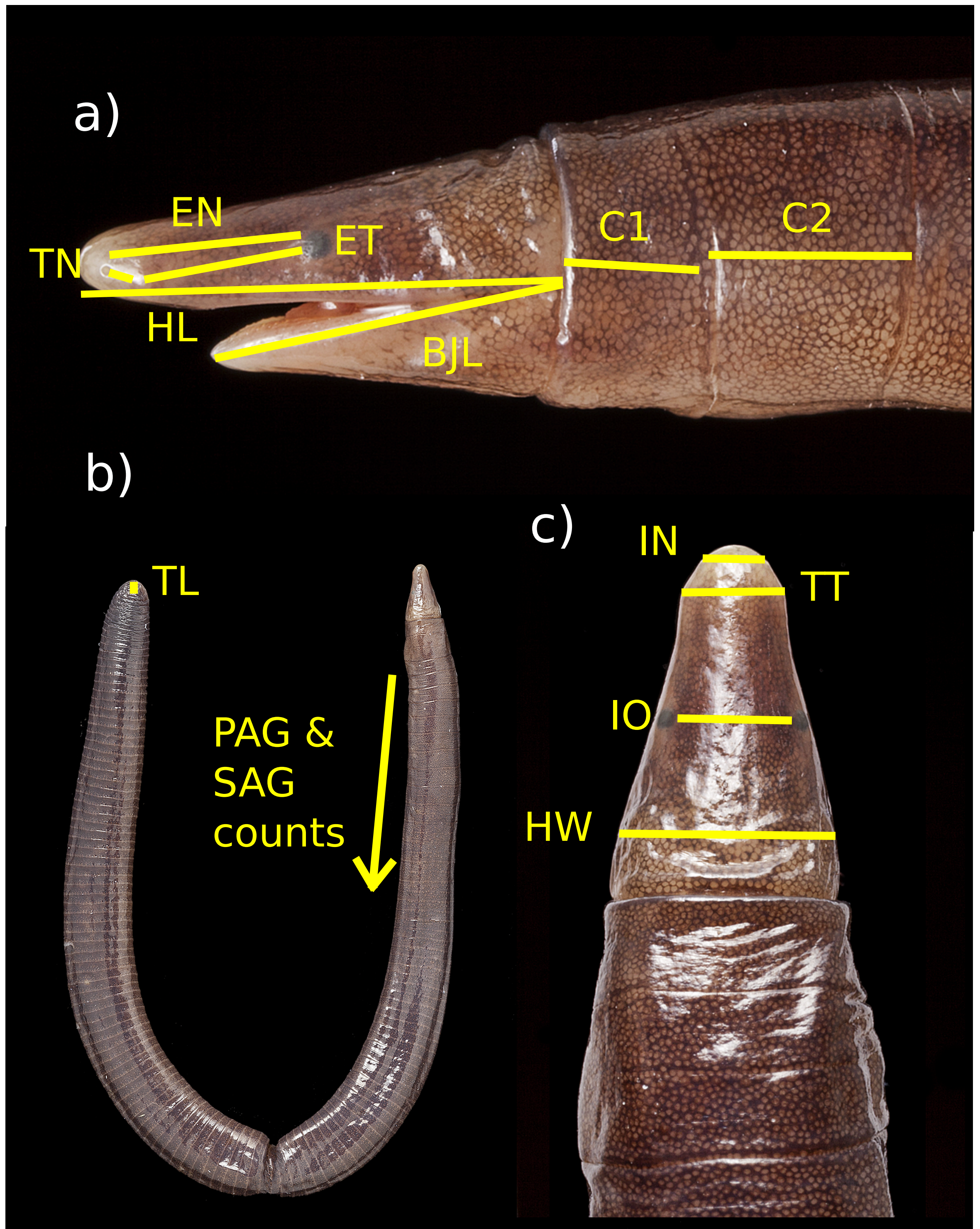

Fig. 2. A *H. brevis* specimen demonstrating the measurements taken on specimens in this chapter: a) lateral head view, b) dorsal body view, c) dorsal head view. Abbreviations used in figure are described in the text (section 3.2).

3.3 Results

3.3.1 Sexual dimorphism

Three species were found to be sexually dimorphic from the MANOVA tests: *G. larvata, G. sechellensis* and *H. rostratus* (Table 1; see Fig. 3). Separate PCA analyses of head measurements were carried out on these three species as they were found to be the most influenced by sexual dimorphism (Fig. 4). The first two principal components accounted for 91.6% (PC1 84.1%, PC2 7.5%) of the variation in *G. larvata*, 93% in *G. sechellensis* (PC1 85%, PC2 8%) and 92.3% (PC1 86%, PC2 6.3%) in *H. rostratus*. In *G. larvata* and *G. sechellensis* sexual dimorphism was evident from the PCA plots. On the PC1 axes higher values are indicative of longer and wider heads and longer lower jaws – specimens with these attributes tend to be males. Some variation in the PC2 loadings is evident: for *G. larvata* a negative value indicates shorter heads and lower jaws whereas a positive value is associated with greater head width and tentacle-tentacle distance. Within *G. sechellensis* a negative score is generally reflected in shorter heads and lower jaws, shorter distances between eye and naris, and between tentacle and naris, whereas those individuals with a positive value tend to have a wider head and greater distances between the orbits and between the tentacles.

The PCA plot for all *H. rostratus* does not clearly separate males and females, but there is evidence of sexual dimorphism when specimens from individual islands are examined separately (Fig. 4), with males tending to have larger head measurements than females. High PC1 scores are indicative of greater head lengths and widths and distances between eyes and between eye and naris. Negative PC2 values are associated with shorter heads and

positive values associated with greater head widths and distances between the eyes.

Thus, head dimensions are sexually dimorphic in *G. larvata* and *G. sechellensis,* and to a lesser extent, in *H. rostratus*. These differences between the sexes are most prominent in *G. sechellensis* where female heads are considerably smaller than males (Fig. 3). In contrast, no evidence was found for sexual dimorphism in *G. alternans, P. cooperi,* and the *H. brevis* group.

Table 1 MANOVA results for variation observed within each species of Seychelles caecilian and between the sexes of each species.

	Between islands				Sexual dimorphism			
	Wilks' lambda	*F*	df (1, 2)	*p*	Wilks' lambda	*F*	df (1, 2)	*p*
G. alternans	0.03693	3.628	100, 375.4	<0.005	0.8484	0.6771	19, 72	0.829
G. larvata	0.09225	1.854	60, 167.7	<0.005	3.16E-261	5.00E+260	19, 30	<0.005
G. sechellensis	0.1178	2.105	40, 44	0.008485	0.09363	9.681	20, 20	<0.005
H. brevis group	0.02808	11.8	32, 76	<0.005	0.6923	0.6012	17, 23	0.8574
H. rostratus	0.1289	13.44	56, 1626	<0.005	0.878	5.331	8, 307	<0.005
P. cooperi	0.2103	1.502	10, 4	0.37	0.6158	0.2496	10, 4	0.9658

Table 2 Mean and standard deviation of morphometric and meristic data for *Grandisonia alternans* grouped by island. Characters TL, BW/BC, HL, BJL, HW, IO, IN, EN, ET, TN, C1, C2, TT and Tail length are in mm. The remaining characters are counts. Number of specimens per island are displayed in the first row. See Materials and Methods for explanation of abbreviations.

	Mahé (*n* = 24)		Silhouette (*n* = 34)		Praslin (*n* = 3)		La Digue (*n* = 5)		Frégate (*n* =28)		Félicité (*n* = 7)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TL	200.21	70.86	222.18	15.38	250.33	18.5	214.6	39.45	246.11	33.29	199.86	47.84
BW/BC	31	11.66	35.65	2.93			37.33	13.32	40.21	8.19	29.86	5.81
HL	9.8	2.84	10.03	0.77	10.43	0.57	9.38	1.2	10.66	1.18	9.03	2.17
BJL	8.84	2.73	9.06	0.74	9.37	0.42	8.44	1.17	9.51	1.15	8.06	2.1
HW	7.33	2.4	7.95	0.61	7.83	0.06	7.78	1.16	8.61	1.1	7.3	1.57
IO	4.37	1.44	4.68	0.29	4.7	0.1	4.42	0.64	5.12	0.64	4.23	0.89
IN	1.81	0.47	1.88	0.15	2.03	0.06	1.82	0.22	2.06	0.27	1.77	0.41
EN	3.46	1.06	3.44	0.28	3.6	0.1	3.38	0.42	3.81	0.52	3.27	0.77
ET	2	0.78	1.91	0.2	1.93	0.15	1.82	0.23	2.13	0.3	1.66	0.45
TN	1.5	0.46	1.66	0.14	1.63	0.12	1.54	0.23	1.67	0.24	1.66	0.37
C1	2.98	0.98	3.2	0.24	3.33	0.21	3.08	0.62	3.54	0.5	3	0.63
C2	3.08	1.05	4.15	4.58	3.23	0.25	3.14	0.58	3.78	0.52	3.13	0.78
TT	4.02	1.27	4.42	0.32	4.17	0.06	4.12	0.63	4.68	0.57	4.11	0.85
Tail length	2.7	0.76	2.88	0.37	3	0.44	3	0.54	2.94	0.51	2.49	0.4
C1 TG	0.17	0.38	0.18	0.39	0	0	0.2	0.45	0.07	0.26	0.29	0.49
C2 TG	1.46	0.83	1.24	0.55	1	0	1	0	1.29	0.6	1.29	0.49
PAG	84.58	1.28	82.71	2.13	85.33	0.58	84.6	1.67	83.29	2.65	83.71	1.5
dSAG	0.21	0.59	0.06	0.24	8.33	5.51	1.8	2.39	0.11	0.31	0.71	0.76
fcSAG	38.04	13.91	38.35	11.36	58	7	52.4	2.41	42.79	11.97	48.14	6.41
vSAG	62.88	6.36	50.53	7.3	72.33	4.16	69.8	4.32	61.57	5.96	65.43	5.77
VAG	4.04	0.69	4.24	0.55	4	0	4.2	0.45	5.04	0.59	4	0

Table 3 Mean and standard deviation of morphometric and meristic data for males of *Grandisonia larvata* grouped by island. Characters TL, BW/BC, HL, BJL, HW, IO, IN, EN, ET, TN, C1, C2, TT and Tail length are in mm. The remaining characters are counts. Number of specimens per island are displayed in the first row. See Materials and Methods for explanation of abbreviations.

	Mahé (*n* = 6)		Silhouette (*n* = 5)		Praslin (*n* = 2)		La Digue (*n* = 5)		Félicité (*n* = 3)		Ste Anne (*n* = 1)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TL	166.33	26.31	194.20	12.38	149.50	6.36	175.60	16.01	203.00	26.63	204	
BW/BC	25.67	3.50	29.20	4.27	24.50	2.12	25.40	2.07	29.33	3.21	32	
HL	7.07	1.21	8.04	0.83	5.75	0.21	6.68	1.03	8.53	1.53	8.4	
BJL	6.18	1.12	6.90	0.75	4.90	0.28	5.92	1.01	7.37	1.50	7.1	
HW	5.07	0.83	5.86	0.50	4.30	0.42	4.98	0.38	5.83	1.12	6.2	
IO	2.97	0.43	3.60	0.23	2.55	0.07	2.88	0.29	3.37	0.74	3.7	
IN	1.30	0.19	1.50	0.10	1.15	0.21	1.20	0.10	1.47	0.21	1.4	
EN	2.77	0.57	3.30	0.32	2.20	0.14	2.74	0.52	3.23	0.78	3.4	
ET	1.67	0.38	1.86	0.27	1.25	0.07	1.56	0.34	1.87	0.47	2.1	
TN	1.20	0.25	1.44	0.11	0.90	0.14	1.22	0.22	1.40	0.36	1.4	
C1	2.35	0.22	2.52	0.30	1.95	0.07	2.18	0.29	2.80	0.72	2.4	
C2	2.42	0.38	2.76	0.44	1.95	0.21	2.34	0.41	2.83	0.42	2.4	
TT	2.73	0.39	3.42	0.26	2.15	0.07	2.62	0.18	3.50	0.92	3.6	
Tail length	1.93	0.28	2.46	0.31	1.60	0.14	2.24	0.24	2.07	0.74	2.4	
C1 TG	0	0	0	0	0	0	0	0	0	0	0	
C2 TG	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1	
PAG	76.00	1.10	75.20	1.64	78.00	0.00	78.60	1.14	79.33	0.58	81	
dSAG	19.83	2.14	15.60	4.16	21.50	0.71	19.40	2.07	22.67	0.58	20	
fcSAG	41.33	2.42	37.40	1.67	47.50	2.12	43.80	3.19	48.67	1.53		
vSAG	51.33	3.39	44.60	3.21	55.50	0.71	51.60	3.36	55.00	2.65	46	
VAG	2.67	0.82	3.60	0.55	3.00	0.00	3.80	0.45	3.00	0.00	56	

Table 4 Mean and standard deviation of morphometric and meristic data for females of *Grandisonia larvata* grouped by island. Characters TL, BW/BC, HL, BJL, HW, IO, IN, EN, ET, TN, C1, C2, TT and Tail length are in mm. The remaining characters are counts. Number of specimens per island are displayed in the first row. See Materials and Methods for explanation of abbreviations.

	Mahé (*n* =8)		Silhouette (*n* =1)		Praslin (*n* = 2)		La Digue (*n* = 9)		Félicité (*n* = 4)		Ste Anne (*n* = 3)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TL	158.25	20.64	191.00		186.00	1.41	177.67	23.17	210.00	20.93	186.00	24.25
BW/BC	26.75	3.41	31.00		29.50	3.54	27.33	4.09	33.00	4.24	30.00	2.65
HL	6.40	0.52	7.60		6.65	0.21	6.34	0.74	7.53	0.70	7.00	1.21
BJL	5.40	0.35	6.20		5.80	0.28	5.37	0.38	6.50	0.74	6.03	1.12
HW	4.51	0.44	4.90		5.20	0.28	4.74	0.59	5.63	0.62	5.20	0.85
IO	2.80	0.25	3.20		2.85	0.07	2.73	0.30	3.15	0.30	3.10	0.50
IN	1.20	0.13	1.60		1.30	0.00	1.21	0.11	1.40	0.18	1.33	0.21
EN	2.41	0.21	2.60		2.65	0.07	2.49	0.26	2.93	0.30	2.80	0.56
ET	1.40	0.17	1.60		1.50	0.00	1.41	0.19	1.58	0.19	1.60	0.35
TN	1.05	0.11	1.20		1.10	0.00	1.12	0.11	1.38	0.13	1.27	0.21
C1	2.18	0.17	2.80		2.35	0.07	2.16	0.26	2.63	0.38	2.03	0.46
C2	2.19	0.24	2.70		2.45	0.07	2.30	0.27	2.65	0.38	2.13	0.55
TT	2.54	0.22	2.70		2.55	0.07	2.54	0.26	3.08	0.40	2.80	0.46
Tail length	1.95	0.34	2.50		2.00	0.57	1.99	0.33	2.05	0.30	1.83	0.06
C1 TG	0.00	0.00			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C2 TG	0.75	0.46			0.50	0.71	1.00	0.00	1.00	0.00	1.00	0.00
PAG	76.38	1.19	72.00		77.00	0.00	79.22	1.20	79.00	0.82	80.67	1.15
dSAG	12.38	8.65	24.00		18.50	2.12	21.22	1.64	23.50	1.00	18.67	1.53
fcSAG	40.63	3.78	43.00		42.00	1.41	45.56	1.88	45.75	1.50	46.67	3.06
vSAG	53.00	3.07	58.00		52.50	3.54	54.78	4.02	54.00	1.41	57.00	1.73
VAG	3.38	0.52	4.00		3.50	0.71	3.89	0.33	3.00	0.00	3.67	0.58

Table 5 Mean and standard deviation of morphometric and meristic data for males of *Grandisonia sechellensis* grouped by island. Characters TL, BW/BC, HL, BJL, HW, IO, IN, EN, ET, TN, C1, C2, TT and Tail length are in mm. The remaining characters are counts. Number of specimens per island are displayed in the first row. See Materials and Methods for explanation of abbreviations.

	Mahé (*n* = 8)		Silhouette (*n* = 9)		Praslin (*n* = 1)	
	Mean	SD	Mean	SD	Mean	SD
TL	197.88	21.09	179.22	17.96	206	
BW/BC	28.13	4.05	28.44	3.28	32	
HL	7.36	0.70	6.87	0.64	7.5	
BJL	6.58	0.59	6.22	0.55	6.7	
HW	5.30	0.66	5.10	0.39	5.4	
IO	2.93	0.27	2.87	0.24	3.5	
IN	0.94	0.11	0.89	0.13	1.1	
EN	3.01	0.23	2.82	0.28	3.3	
ET	0.99	0.10	0.87	0.09	1	
TN	2.05	0.18	1.91	0.19	2.3	
C1	2.26	0.26	1.92	0.21	2	
C2	2.53	0.35	2.39	0.29	2.5	
TT	3.34	0.44	3.14	0.30	3.6	
Tail length	1.91	0.29	1.76	0.44	2	
C1 TG	0.25	0.46	0.11	0.33	0	
C2 TG	0.88	0.35	0.67	0.50	1	
PAG	82.00	1.51	80.56	1.59	82	
dSAG	14.50	3.25	9.44	6.82	17	
fcSAG	33.75	4.10	34.11	5.13	42	
vSAG	50.50	7.05	51.44	6.52	55	
VAG	3.00	0.76	1.89	0.33	3	

Table 6 Mean and standard deviation of morphometric and meristic data for females of *Grandisonia sechellensis* grouped by island. Characters TL, BW/BC, HL, BJL, HW, IO, IN, EN, ET, TN, C1, C2, TT and Tail length are in mm. The remaining characters are counts. Number of specimens per island are displayed in the first row. See Materials and Methods for explanation of abbreviations.

	Mahé (*n* = 11)		Silhouette (*n* = 11)		Praslin (*n* = 1)	
	Mean	SD	Mean	SD	Mean	SD
TL	181.55	12.66	188.91	15.94	181.55	
BW/BC	27.73	2.33	31.18	2.40	27.73	
HL	6.15	0.42	6.18	0.23	6.15	
2BJL	5.46	0.41	5.57	0.20	5.46	
HW	4.45	0.34	4.62	0.33	4.45	
IO	2.45	0.18	2.56	0.14	2.45	
IN	0.78	0.09	0.82	0.09	0.78	
EN	2.51	0.20	2.53	0.13	2.51	
ET	0.69	0.09	0.76	0.07	0.69	
TN	1.72	0.17	1.72	0.11	1.72	
C1	2.05	0.20	1.96	0.17	2.05	
C2	2.25	0.16	2.42	0.25	2.25	
TT	2.80	0.24	2.85	0.16	2.80	
Tail length	1.58	0.46	1.69	0.36	1.58	
C1 TG	0.18	0.40	0.18	0.40	0.18	
C2 TG	1.10	0.74	1.09	0.30	1.10	
PAG	83.91	1.92	82.91	0.94	83.91	
dSAG	14.45	7.54	13.64	2.62	14.45	
fcSAG	36.27	5.41	34.27	9.57	36.27	
vSAG	54.82	5.13	49.36	4.48	54.82	
VAG	2.91	0.70	2.30	0.48	2.91	

Table 7 Mean and standard deviation of morphometric and meristic data for the *Hypogeophis brevis* group grouped by island. Characters TL, BW/BC, HL, BJL, HW, IO, IN, EN, ET, TN, C1, C2, TT and Tail length are in mm. The remaining characters are counts. Number of specimens per island are displayed in the first row. See Materials and Methods for explanation of abbreviations.

	brevis (*n* = 39)		Congo Rouge (*n* = 5)		Praslin (*n* = 15)	
	Mean	SD	Mean	SD	Mean	SD
TL	79.97	22.44	90.8	10.11	71.43	21.29
BC	15.53	4.96	15.4	0.89	15.8	5.56
HL	3.77	0.43	4.62	0.30	4.01	0.63
BJL	2.92	0.34	3.72	0.28	3.1	0.53
HW	2.52	0.42	2.84	0.24	2.65	0.49
IO	1.44	0.22	1	0.07	1.45	0.27
IN	0.59	0.1	2.44	0.05	0.57	0.12
EN	1.61	0.24	1.34	0.11	1.67	0.26
ET	1.33	0.23	0.76	0.11	0.93	0.19
TN	0.27	0.06	1.52	0.08	0.72	0.09
C1	1.12	0.19	1.24	0.09	1.12	0.23
C2	1.43	0.34	1.38	0.16	1.36	0.3
TT	1.01	0.13	1.1	0.16	1.21	0.21
Tail length	1.09	0.27	1.14	0.15	1.04	0.22
C1 TG	0	0	0	0	0.07	0.26
C2 TG	1.18	0.56	1.4	0.89	1.6	0.63
PAG	65.62	1.41	69.6	0.55	62.21	0.89
dSAG	2.74	2.97	0	0.00	0	0
fcSAG	14.21	6.27	11.4	6.50	0.67	1.91
vSAG	21.36	5.07	16.2	6.98	19.53	8.47
VAG	1.22	0.68	1.8	0.84	1.64	0.63

Table 8 Mean and standard deviation of morphometric and meristic data for males of *Hypogeophis rostratus* grouped by island. Characters TL, BW, HL, HW, IO, IN, EN, ET, TN are in mm. The remaining characters are counts. Number of specimens per island are displayed in the first row. See Materials and Methods for explanation of abbreviations.

	Mahé (*n* = 51)		Silhouette (*n* = 26)		Praslin (*n* = 23)		La Digue (*n* = 29)		Frégate (*n* = 18)		Félicité (*n* = 9)		Ste Anne (*n* = 8)		Curieuse (*n* = 5)	
	Mean	SD	Mean	SD	Mean	Mean	SD	Mean	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TL	226.67	51.29	208.42	40.99	221.17	41.68	294.76	48.82	245.33	68.72	228.11	32.01	257.75	31.49	287.00	20.92
BW	7.98	1.82	8.08	1.49	8.26	1.63	11.72	2.33	10.39	3.22	9.11	1.54	9.38	1.41	10.40	1.52
HL	7.79	1.54	7.40	1.21	6.38	0.96	8.64	1.44	7.81	1.69	6.79	0.70	7.96	1.17	7.98	0.61
HW	5.40	1.15	4.88	0.86	4.30	0.86	6.10	1.19	5.74	1.65	4.67	0.63	6.01	1.15	5.90	0.54
IO	3.73	0.91	3.44	0.70	2.94	0.63	4.34	0.89	4.14	1.31	3.14	0.49	4.06	0.79	4.02	0.36
IN	1.82	0.46	1.69	0.36	1.70	1.62	2.17	0.47	1.86	0.55	1.64	0.20	1.89	0.36	2.08	0.19
EN	3.40	0.76	3.11	0.57	2.55	0.49	3.67	0.69	3.33	0.93	2.82	0.32	3.69	0.64	3.32	0.33
ET	2.56	0.66	2.32	0.48	1.91	0.42	2.87	0.62	2.45	0.77	2.08	0.32	2.83	0.64	2.60	0.32
TN	1.15	0.25	1.34	1.37	0.80	0.22	1.51	1.27	1.14	0.31	0.99	0.12	1.16	0.21	1.08	0.16
PAGs	99.86	1.67	97.54	1.70	102.87	1.82	101.03	1.45	95.89	2.00	100.89	0.93	99.88	1.89	105.40	1.14
dSAGs	81.29	7.38	87.54	4.79	92.43	4.65	90.10	4.86	86.50	2.98	91.33	2.35	85.13	4.64	93.40	3.05
vSAGs	96.08	2.60	94.77	1.61	100.78	2.52	98.21	2.43	93.06	2.36	98.44	2.19	97.50	2.83	102.80	2.59
VAG	2.22	0.67	1.35	0.63	1.91	0.67	2.03	0.57	2.00	0.69	2.67	0.50	1.38	0.52	2.20	0.45

Table 9 Mean and standard deviation of morphometric and meristic data for females of *Hypogeophis rostratus* grouped by island. Characters TL, BW, HL, HW, IO, IN, EN, ET, TN are in mm. The remaining characters are counts. Number of specimens per island are displayed in the first row. See Materials and Methods for explanation of abbreviations.

	Mahé (*n* = 55)		Silhouette (*n* = 15)		Praslin (*n* = 18)		La Digue (*n* = 16)		Frégate (*n* = 21)		Félicité (*n* = 11)		Ste Anne (*n* = 9)		Curieuse (*n* = 1)	
	Mean	SD	Mean	SD	Mean	Mean	SD	Mean	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TL	224.33	48.31	196.93	35.03	199.67	27.88	275.81	37.18	241.57	44.58	230.73	25.31	252.22	36.84	251.00	
BW	8.27	2.09	7.67	1.35	7.39	1.29	11.13	2.25	10.76	2.49	9.73	1.62	9.78	1.92	11.00	
HL	7.23	1.08	6.76	0.83	5.69	0.58	7.55	0.93	7.44	0.85	6.60	0.42	7.23	1.75	6.60	
HW	5.07	0.95	4.59	0.57	3.79	0.51	5.16	0.72	5.35	0.99	4.50	0.46	5.57	0.78	5.00	
IO	3.43	0.63	3.17	0.44	2.54	0.38	3.66	0.48	3.77	0.66	3.01	0.28	3.80	0.55	3.40	
IN	1.68	0.31	1.53	0.19	2.10	2.52	1.83	0.25	1.65	0.27	1.60	0.12	1.73	0.25	1.60	
EN	3.09	0.56	2.84	0.35	2.23	0.32	3.16	0.42	2.94	0.44	2.70	0.24	3.30	0.46	2.80	
ET	2.28	0.47	2.08	0.32	1.63	0.30	2.42	0.40	2.18	0.38	1.93	0.20	2.47	0.39	2.10	
TN	1.01	0.18	1.00	0.10	0.75	0.14	1.49	1.74	1.01	0.17	0.98	0.12	1.10	0.15	1.00	
PAGs	100.71	1.88	97.40	2.26	102.89	2.40	101.00	1.26	95.00	1.92	101.73	1.56	98.89	2.20	105.00	
dSAGs	82.62	8.36	87.07	8.87	92.67	4.93	90.56	2.99	86.38	2.58	92.36	2.11	81.11	5.04	93.00	
vSAGs	97.27	2.44	94.93	3.31	100.28	2.74	98.00	2.45	92.24	2.49	99.27	2.24	95.11	3.33	102.00	
VAG	2.33	0.64	1.73	0.59	1.83	0.51	2.19	0.54	2.24	0.54	2.64	0.50	1.78	0.44	3.00	

Table 10 Mean and standard deviation of morphometric and meristic data for females of *Praslinia cooperi* grouped by island. Characters TL, BW, HL,HW, IO, IN, EN, ET, TN, C1, C2, TT and Tail length are in mm. The remaining characters are counts. Number of specimens per island are displayed in the first row. See Materials and Methods for explanation of abbreviations.

	Mahé (*n* = 8)		Silhouette (*n* = 7)	
	Mean	SD	Mean	SD
TL	200.25	39.09	181.57	28.31
BW/BC	26.38	3.16	26.43	2.99
HL	10.48	1.98	10.61	1.17
BJL	10.2	1.91	10.24	1.16
HW	7.7	1.4	7.69	0.97
IO	4.98	1.02	4.89	0.64
IN	1.94	0.32	1.86	0.27
EN	4.2	1.03	4.3	0.69
ET	0	0	0	0
TN	4.03	1.03	4.1	0.68
C1	3.29	0.58	3.54	0.41
C2	2.88	0.57		
TT	5.73	1.11		
Tail length	2.41	0.55		
C1 TG	0	0	0	0
C2 TG	0.63	0.74		
PAGs	82.13	0.99		
dSAGs	14.13	4.82		
fcSAGs	31.75	2.12		
vSAGs	63.13	5.03		
VAG	3.88	0.64		

3.3.2 Geographic morphometric variation

The PCA analyses of morphometric data failed to support independent clustering of any single-island populations for any species (Fig. 4, 5). In the *H. brevis* group the first two principal component (PC) axes accounted for 65.3% (PC1 = 46.1%, PC2 = 19.2%, PC3 = 8.4%, PC4 = 6.7%, PC5 = 4.7%, PC6 = 4.2%, PC7 = 3.3%) of the variation observed. Specimens with a positive PC1 score tend to have longer heads and lower jaws, greater head width, and greater distance between tentacle and naris. Specimens with a negative PC1 score have a larger distance between eye and tentacle. Positive values in PC2 are dominated by a larger distance between eye and tentacle. A negative score in PC2 mainly reflects a greater distance between the tentacle and nostril. In all analyses the historical specimen, BMNH1987.2109, with no locality data clusters with the population from Praslin (Fig. 5). There is some overlap between *H. brevis sensu stricto* and *H.* cf. *brevis* CR although specimens from Congo Rouge tend to have lower PC1 and PC2 values (Fig. 5). *Hypogeophis* cf*. brevis* Praslin is distinct from the other two members of the *H. brevis* group having higher PC1 scores.

The first two PC axes account for 70% (PC1 = 56.7%, PC2 = 13.3%, PC3 = 8.8%, PC4 = 5.5%, PC5 = 3.6%, PC6 = 2.6%, PC7 = 2.1%) of the variation within *G. alternans*. Longer heads and lower jaws have the greatest weight with positive PC1 scores. In PC2 positive scores are mostly associated with wider heads and longer tails, whereas negative scores are mostly associated with shorter heads and lower jaws. Although there is considerable overlap between populations there is moderate clustering of a southern-island group, and a Frégate + northern-island group, with Frégate specimens slightly

overlapping with those of the southern group. The Frégate + northern-island group tend to have lower PC1 scores (Fig. 5).

Within *G. larvata* the first two PC axes represent 81.5% (PC1 = 71.8%, PC2 = 9.7%, PC3 = 6.5%, PC4 = 2.9%, PC5 = 2.7%, PC6 = 2.1%, PC7 = 1.5%) of the variation in males and 81.4% (PC1 = 71.8%, PC2 = 9.6%, PC3 = 7.8%, PC4 = 3.3%, PC5 = 2.5%, PC6 = 1.5%, PC7 = 1.2%) in females. Longer heads and lower jaws have the highest scores on PC1 in both males and females, but distances between eye-naris and between tentacles in males, and distances between eyes and between tentacles in females also contribute substantially. Positive PC2 scores are predominantly representative of greater head width and distances between the tentacles; in males greater eye-eye distance also contributes considerably. Negative PC2 scores are representative of specimens with shorter heads and lower jaws.

In the combined sex analysis of *G. larvata* there is a small amount of geographic structuring whereby specimens from northern and from southern islands are somewhat distinct, although there is a lot of overlap (Fig. 4). A similar geographic pattern is observed in males and females when analysed separately (Fig. 5). In females a cluster consisting of the northern islands of Praslin, La Digue and Félicité is observed but with considerable overlap with the southern population of Ste Anne. Specimens from the northern-island group generally have smaller head dimensions than those of southern-islands (Fig. 5).

In *G. sechellensis* the first two PC axes represent 74.4% (PC1 = 53%, PC2 = 21.4%, PC3 = 8.8%, PC4 = 5.6%, PC5 = 4.6%, PC6 = 3.2%, PC7 = 1.5%) of the variation in males and 72.5% (PC1 = 44.5%, PC2 = 28%, PC3 =

7.2%, PC4 = 5.8%, PC5 = 5.2%, PC6 = 4%, PC7 = 2.1%) in females. A longer head and lower jaw accounts for the majority of the positive variation in PC1, but distance between eye and naris also contributes extensively as does distance between tentacles in males and tentacle-naris distance in females. Negative PC1 scores reflect a narrower head in females. Positive scores in PC2 are predominantly indicative of wider heads. Negative PC2 values in males are mostly associated with shorter head and lower jaws whereas in females they reflect a shorter distance between eye and naris and between tentacle and naris.

No geographic structuring was observed in *G. sechellensis* though one female specimen from Praslin is distinct from the southern-island specimens (Fig. 4, 5), although this was the only Praslin female specimen included.

The first two PC axes in *H. rostratus* represent 93% (PC1 = 86.6%, PC2 = 6.5%, PC3 = 2.7%, PC4 = 1.9%, PC5 = 1.1%, PC6 = 0.8%, PC7 = 0.5%) of the variation in males and 89.9% (PC1 = 83.1%, PC2 = 6.8%, PC3 = 4.1%, PC4 = 2.9%, PC5 = 1.4%, PC6 = 1.1%, PC7 = 0.6%) in females. The highest scores in PC1 are indicative of a larger heads and greater distance between eyes and between eye and naris. Higher PC1 scores in females are also associated with greater head width. In PC2 the highest negative scores are representative of shorter heads in males and narrower heads and shorter eye-eye distance in females. Positive scores in PC2 are indicative of a wider heads and eye-eye distance in males, and larger heads and greater distances between eye and naris and between eye and tentacle in females.

In all analyses for *H. rostratus,* whether both sexes are combined (Fig. 4, 5) or analysed separately (Fig. 5), a geographic northern vs. southern island

split is observed. Specimens from Frégate occupy an intermediate position between these two main groups (northern-island specimens have lower and southern-island specimens higher PC1 scores). In the combined sex analysis the samples from Frégate occupy a more central position (Fig. 4), but when sexes are analysed separately (Fig. 5) they are more similar to specimens from the southern islands.

In *Praslinia cooperi* the first two PC axes account for 95.6% (PC1 = 79.4%, PC2 = 16.2%, PC3 = 2.4%, PC4 = 0.8%, PC5 = 0.5%, PC6 = 0.4%, PC7 = 0.2%) of the variation within the species. Head and lower jaw length have the highest loading in PC1 with greater lengths having higher PC scores. Negative values in PC2 are more indicative of shorter heads and lower jaws whereas positive values are influenced mostly by greater distance between tentacle and nostril and longer first collars. No geographic pattern is observed in morphological variation in this species (Fig. 4).

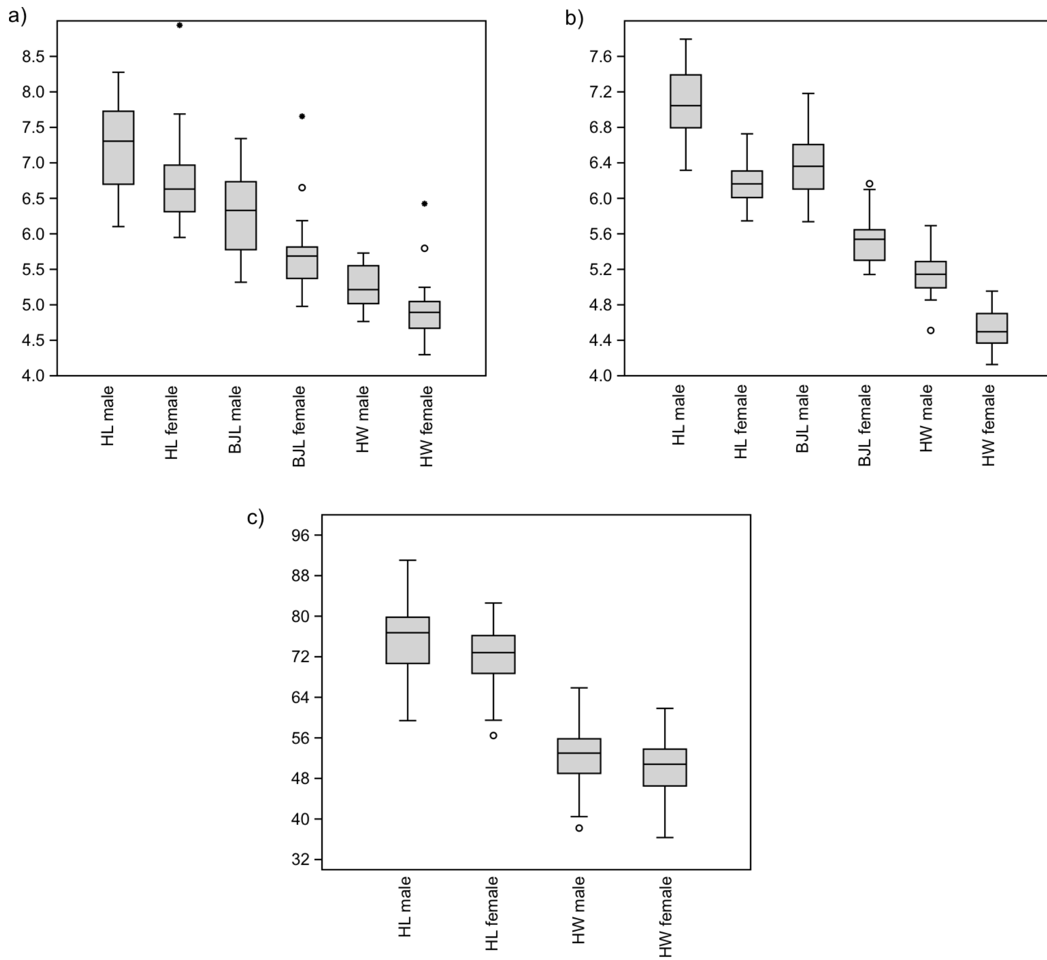

Fig. 3 Boxplots for head dimensions in Seychelles caecilian species showing sexual dimorphism: a) *Grandisonia larvata*; b) *G. sechellensis*; c) *Hypogeophis rostratus*. Numbers on the Y axes are transformed lengths from total body length.

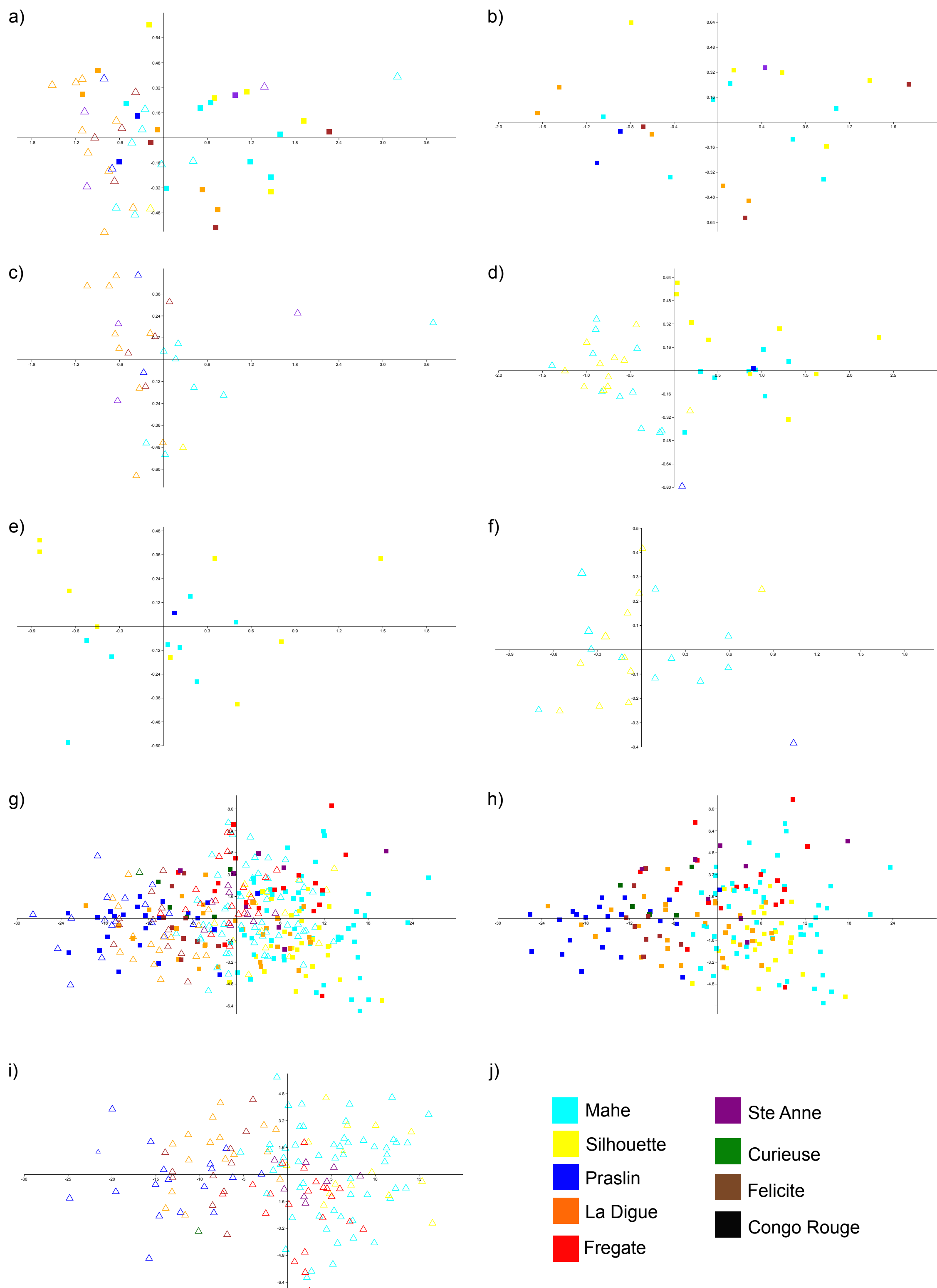

Fig. 4 PCA plots for morphometric characters of the head in species showing sexual dimorphism (*Grandisonia larvata, G. sechellensis* and *Hypogeophis rostratus*) (In combined male and female plots triangles are unfilled to make distinguishing between the sexes easier): a) male and female *G. larvata*; b) male *G. larvata*; c) female *G. larvata*; d) male and female *G. sechellensis*; e) male *G. sechellensis*; f) female *G. sechellensis*; g) male and female *H. rostratus*; h) male *H. rostratus*; i) female *H. rostratus*; j) colour key for island localities used in figure. Triangles = females, squares = males. In all PCA plots horizontal axis is PC1 and vertical axis is PC2.

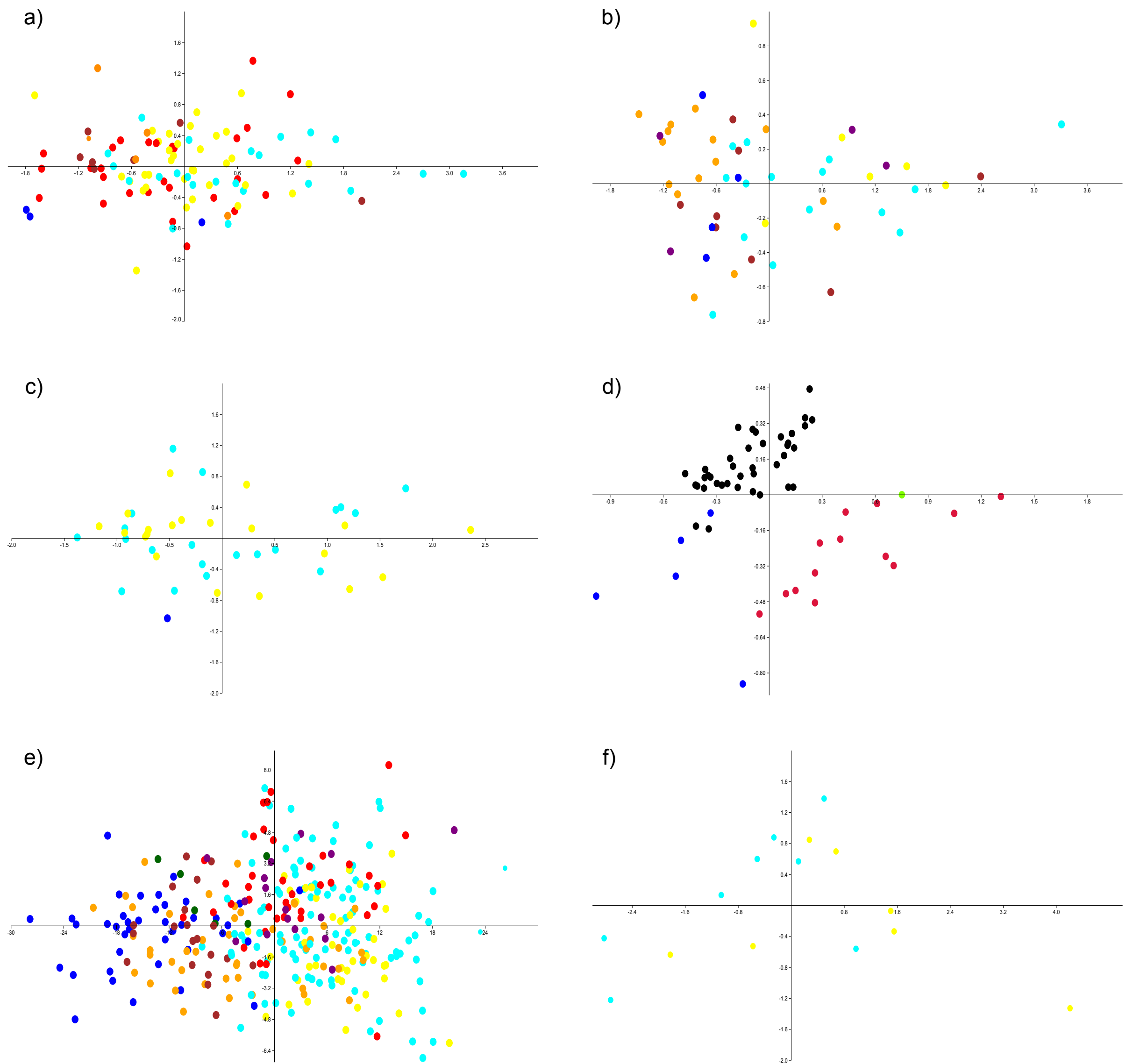

Fig. 5 PCA plots for morphometric characters in all species (and not discriminating between the sexes): a) *Grandisonia alternans*; b) *G. larvata*; c) *G. sechellensis*; d) *Hypogeophis brevis*; e) *H. rostratus*; f) *Praslinia cooperi*. Colours correspond to locality and are those used in Fig. 4. In all PCA plots horizontal axis is PC1 and vertical axis is PC2.

3.3.3 Morphometric and meristic data combined

There is considerable overlap among specimens from different islands across all species in the PCoA analyses (Fig. 6). Population means and standard deviations are provided for all species in Tables 2 – 10. In the *H. brevis* group the first two PCo axes account for 41.3% (PCo1 = 25.1%, PCo2 = 16.2%, PCo3 = 7.5%, PCo4 = 5.4%, PCo5 = 4.5%, PCo6 = 4%, PCo7 = 3.2%) of the variation observed. There is a small amount of overlap in morphospace between *H.* cf. *brevis* CR and *H.* cf. *brevis* Praslin. *Hypogeophis brevis sensu* stricto does not share its morphospace with either of the other two populations.

In *G. alternans* the first two PCo axes account for 31.5% (PCo1 = 18.5%, PCo2 = 13%, PCo3 = 8.5%, PCo4 = 5.6%, PCo5 = 4.4%, PCo6 = 3.8%, PCo7 = 3.4%) of the variation within the species. An approximate cluster of specimens from northern islands is located more towards the higher end of PC1. There is substantial overlap between specimens from the southern islands of Mahé and Silhouette, and Frégate (Fig. 6).

In *G. larvata* the first two PCo axes represent 57.1% (PCo1 = 40.8%, PCo2 = 16.3%, PCo3 = 10.3%, PCo4 = 7.2%, PCo5 = 3.8%, PCo6 = 3.4%, PCo7 = 2.6%) in males and 48.3% (PCo1 = 31.1%, PCo2 = 17.2%, PCo3 = 14.4%, PCo4 = 7.4%, PCo5 = 5.2%, PCo6 = 4.4%, PCo7 = 3.3%) in females of the variation. In both PC1 and PC2, counts relating to primary and secondary annular grooves have the highest loadings. Both sexes of *G. larvata* show geographic structuring and are somewhat similar. Male specimens from Silhouette are distinct and occupy morphospace at the highest ends of PCo1 and PCo2; the single female specimen from Silhouette

occupies morphospace surrounded by specimens from other islands. A northern-island group consisting of specimens from Felicite and Praslin is present in both sexes; the other northern-island, La Digue, overlaps considerably with specimens from Mahé in males but is more distinct in females with only some specimens sharing morphospace with the southern islands of Mahé and Ste Anne (Fig. 6).

In *G. sechellensis* the first two PCo axes represent 47.5% (PCo1 = 29.4%, PCo2 = 18.1%, PCo3 = 14.2%, PCo4 = 7.9%, PCo5 = 6.8%, PCo6 = 5.5%, PCo7 = 4%) of the variation in males and 42.2% (PCo1 = 24.4%, PCo2 = 17.8%, PCo3 = 12%, PCo4 = 8.1%, PCo5 = 7.2%, PCo6 = 5.4%, PCo7 = 4.3%) in females. No distinct geographic pattern in morphological variation was observed within *G. sechellensis* but the Praslin specimen of each sex is seemingly distinct but because only a single specimen is available for each sex this cannot be confirmed (Fig. 6).

The first two PCo axes in *H. rostratus* represent 74% (PCo1 = 46.1%, PCo2 = 27.9%, PCo3 = 16%, PCo4 = 3.3%, PCo5 = 1.9%, PCo6 = 1.3%, PCo7 = 1.1%) in males and 38.6% (PCo1 = 28.6%, PCo2 = 10%, PCo3 = 6.7%, PCo4 = 5.9%, PCo5 = 3.4%, PCo6 = 2.9%, PCo7 = 2.3%) in females of the variation observed. Variation in both sexes of *H. rostratus* show the same general pattern of geographic structuring with a main split into northern- and southern-island groups. Within the southern-island group, specimens from Mahé and Silhouette are somewhat distinct. Specimens from the eastern island of Frégate overlap with those from Silhouette and the northern island of La Digue. In females specimens from Praslin and La Digue are more distinct than they are in males (Fig. 6).

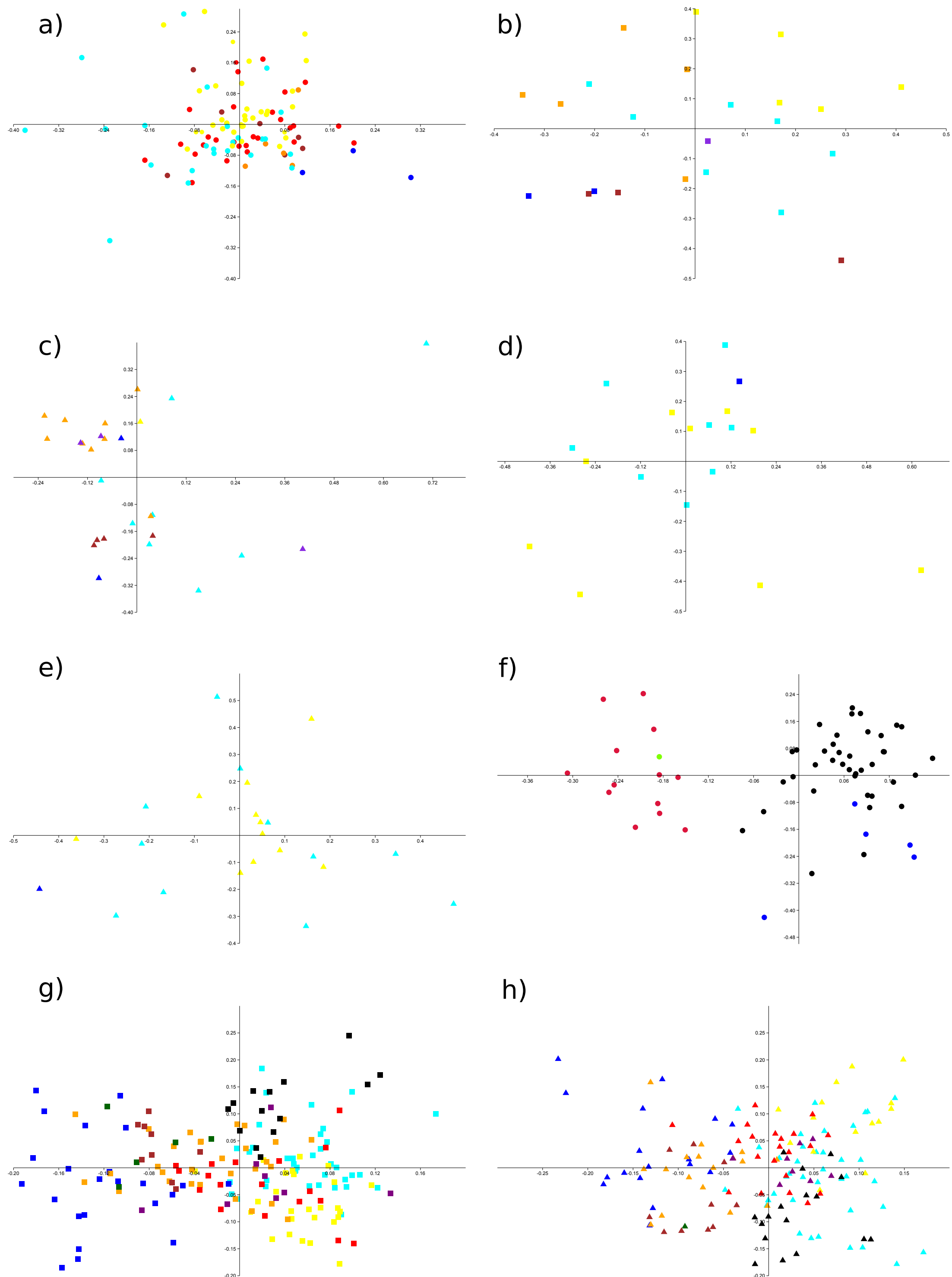

Fig. 6 PCoA plots for morphometric and meristic characters for Seychelles caecilians: a) *Grandisonia alternans*; b) male *G. larvata*; c) female *G. larvata*; d) male *G. sechellensis*; e) female *G. sechellensis*; f) *Hypogeophis brevis* group; g) male *H. rostratus*; h) female *H. rostratus*. Colours correspond to locality and are those used in Fig. 4. Triangles = females, squares = males, circles = combined males and females. In all PCoA plots horizontal axis is PCo1 and vertical axis is PCo2.

3.4 Discussion

3.4.1 Sexual dimorphism

Few studies have studied or reported sexual dimorphism in caecilians (reviewed in Kupfer 2009) and so the present study extends knowledge of sexual dimorphism in these amphibians. The results obtained here identify sexual dimorphism in three of the eight (six nominal and two undescribed) Seychelles caecilian species. One of these had been documented previously in analyses of a different dataset for *Hypogeophis rostratus* (Nussbaum and Pfrender 1998). The two newly reported cases of sexual dimorphism were observed in the sister species *Grandisonia larvata* and *G. sechellensis*. The sister species to *H. rostratus* is *G. alternans* (see Chapter 6) which lacks evidence of sexual dimorphism. It is unlikely that *H. rostratus* shares an especially close relationship with *G. larvata* and *G. sechellensis* (see Chapter 6), thus it is highly likely that sexual dimorphism is homoplastic in Seychelles caecilians. The evidence of multiple gain or loss events (outgroup conditions are not well known) in the evolution of sexual dimorphism among Seychelles caecilians fails to reject the hypothesis that sexual selection may have played an important role in the diversification of this radiation.

The larger head size of males compared to females in *H. rostratus* is consistent with the findings of Nussbaum and Pfrender (1998). As with *H. rostratus,* both *G. larvata* and *G. sechellensis* males have larger head dimensions than females. Head width and length has been found to be dimorphic across four of the ten caecilian families, including in another member of the Indotyphlidae (the family to which the Seychelles caecilians belong) (Presswell 2002) and species of Dermophiidae (Nussbaum & Pfrender

1998; Teodecki *et al.* 1998; Delétre & Measey 2004), Herpelidae (Malonza & Measey 2005; Jones *et al.* 2006) and Scolecomorphidae (Nussbaum 1985). Head size dimorphism in caecilians is thought to be linked with differences in diet, burrowing speed and/or combat but very few thorough studies have been carried out.

In general, sexual dimorphism is thought to evolve in response to sexual selection and/or niche segregation with somewhat different ecologies in each sex (see Berns 2013). Dietary divergence between the sexes in caecilians has received little research but the two studies that have investigated this have reported contrasting results, with differences (in propotions of various dietary components in *Boulengerula boulengeri*: Jones *et al.* 2006) and no differences (*Schistometopum thomense*: Delétre & Measey 2004) detected between the sexes. There are no detailed data on diet in any Seychelles caecilian species. Greater understanding of sexual dimorphism in non-Seychelles caecilians, and of the ecology (including diet) of both in- and outgroup species would permit more detailed evolutionary analyses of this phenomenon in Seychelles caecilians and its possible role in their diversification.

Table 11. Patterns of geographic structuring of morphological variation observed in the Seychelles caecilians (filled grey rectangles indicate that a geographic unit is distinct). The Northern and Southern headings refer to the northern- and southern-island groups (see section 3.2 for explanation of island groups), respectively. If there is substructure in the northern- and southern-island groups then this is indicated in other cells. See Fig. 7 for a graphical representation.

	Northern	**Southern**	**Mahé**	**Silhouette**	**Frégate**	**Praslin**	**Mahé + Frégate**	**No pattern**
G. alternans	■	■		■			■	
G. larvata	■	■	■	■				
G. sechellensis								■
H. brevis group	■	■	■			■		
H. rostratus	■	■			■			
P. cooperi								■

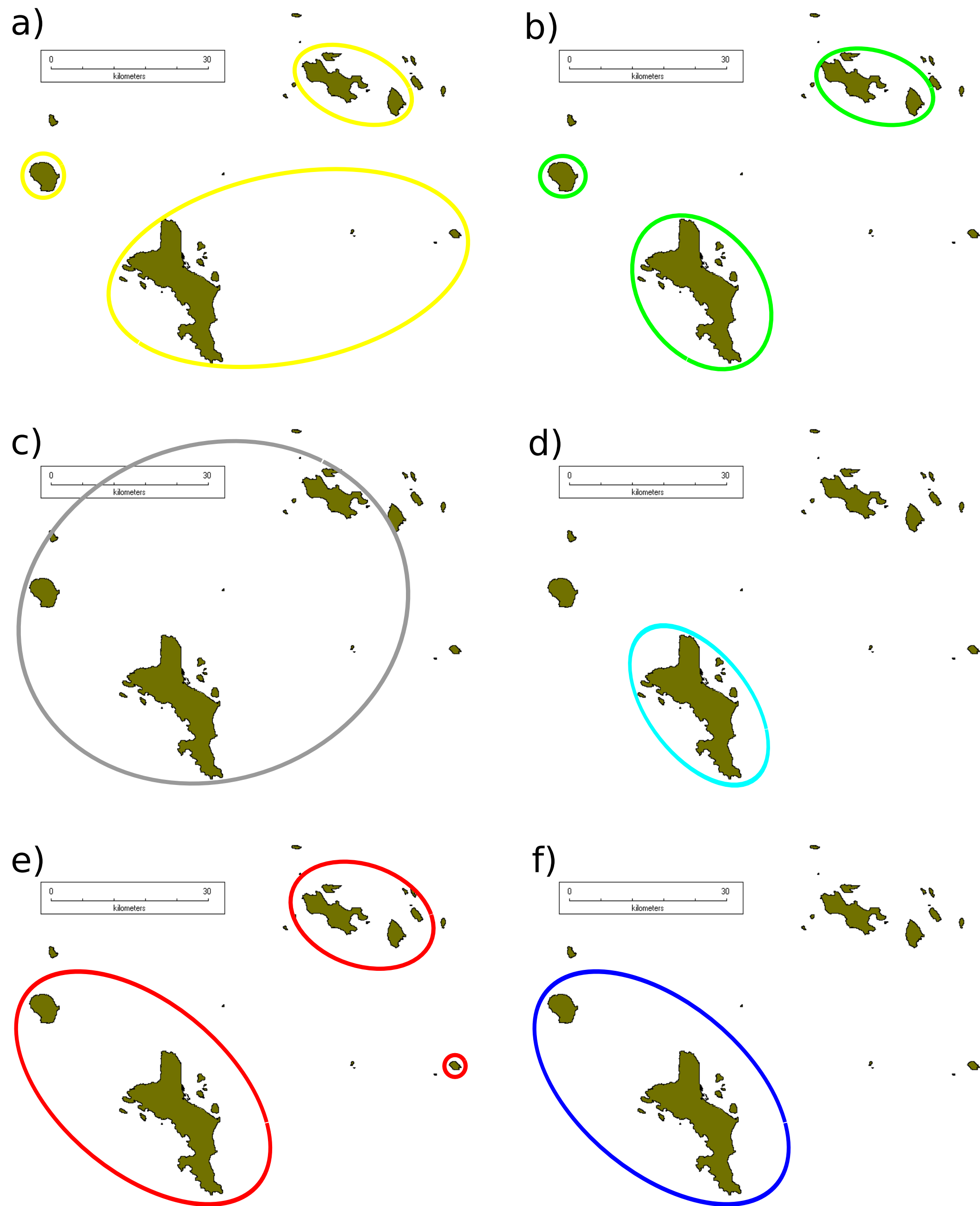

Fig. 7 Maps of the granitic Seychelles showing the patterns of clustering in each species of caecilian studied in this chapter: a) *Grandisonia alternans*, b) *G. larvata*, c) *G. sechellensis*, d) *Hypogeophis brevis*, e) *H. rostratus*, f) *Praslinia cooperi*.

3.4.2 Geographic structuring of morphological variation

The Seychelles caecilian amphibians exhibit differing geographic patterns of phenotypic variation across their ranges, something also found in different groups of Seychelles frogs in phenotypic and molecular data (Nussbaum & Wu 1995; Van Der Meijden *et al.* 2007; Taylor *et al.* 2012; Maddock *et al.* 2014a), but unlike the generally similar patterns found among different Seychelles lizards (Radtkey 1996; Rocha *et al.* 2010a, 2011, 2013b; Valente *et al.* 2014). Two of the five caecilian species that occur across multiple islands, *Praslinia cooperi* and *Grandisonia sechellensis,* showed no clear evidence of geographic structuring of morphological variation. The geographic variation observed between *H. brevis sensu stricto, H*. cf. *brevis* Praslin and *H*. cf. *brevis* CR is here considered inter- rather than intraspecific variation.

The four Seychelles caecilian species that have notably geographically structured morphological variation (*G. alternans, G. larvata, H. brevis* group, and *H. rostratus*), while having somewhat different patterns to each other, all generally have northern- and southern-island clusters (summarised in Table 11). Though few thorough studies investigating morphological patterns of geographic variation in any Seychelles organisms across multiple islands have been carried out (but see Nussbaum & Wu 1995), molecular analyses have generally recovered distinct northern and southern lineages (Radtkey 1996; Rocha *et al.* 2010a, 2011, 2013b; Valente *et al.* 2014). Within the southern-island group, morphological variation in both *G. alternans* and *G. larvata* is further partitioned between specimens from Silhouette and Mahé. This pattern of northern, Silhouette and Mahé(+Frégate) clusters is similar to molecular results reported for a sooglossid frog (Taylor *et al.* 2012) and freshwater crab

(Daniels 2011), and for the latter, each lineage is now recognised as a distinct species (Cumberlidge & Daniels 2014).

Oscillating sea levels may have substantially influenced some of the discontinuities observed in morphology that could perhaps correspond to breaks in gene flow in the Seychelles caecilians. As with other amphibians, caecilian distributions and their dispersal are likely influenced by saltwater barriers due to the osmotic properties of their skin (e.g. Balinsky 1981; Duellman & Trueb 1986) and therefore there is likely to be very little migration between island populations when sea levels are at their present or higher levels. Sea levels have fluctuated extensively over the recent past (Colonna *et al.* 1996; Rohling *et al.* 1998; Siddall *et al.* 2003; Camoin *et al.* 2004; Miller *et al.* 2005) and these oscillations would have resulted in periodic connection of all of the granitic islands (see Fig. 3 in Chapter 1). Only the lowest stands in sea level would have connected the island of Silhouette with the remaining islands and this might explain the patterns observed in *G. alternans* and *G. larvata*. However, abiotic factors might be expected to similarly affect all of the closely related and ecologically superficially similar species, but this is not the case. It is also possible that geographic structure in morphological intraspecific variation in Seychelles caecilians is explained by local adaptation, greater ecological differences than is currently apparent, and/or the effects of small populations on islands rather than substantial breaks in gene flow.

It cannot be ruled out that the variation in geographic patterns of morphological variation observed among the different caecilian species here could be caused by differing ecologies. Very little is still known regarding the ecologies of the Seychelles caecilians with even life-history strategies

remaining unknown for some species (Nussbaum 1984a; San Mauro *et al.* 2014). Unusually for closely related caecilians early life-histories are varied among the Seychelles species. All are oviparous (as far as we are aware (R.A. Nussbaum unpublished; pers. obs.)) but *P. cooperi* has a fully aquatic, extended larval stage; *G. larvata* and *G. sechellensis* have a brief larval stage; and *H. rostratus* has direct development. The early life-history strategies of *G. alternans* and the *H. brevis* group are unknown. Based on the numbers of sampled specimens and lack of detailed information regarding species distributions it is unclear whether patterns of morphological variation may be accelerated in areas where species occur in sympatry by character displacement (Brown & Wilson 1956). Character displacement has been observed in other amphibian species that have partially sympatric distributions (e.g. Loftus-Hill & Littlejohn 1992; Pfennig & Murphy 2000; Adams & Rohlf 2000; Leary 2001) but never in caecilians.

The analyses presented in this chapter have uncovered interesting patterns of intraspecific variation that now place greater emphasis on the need for generating better ecological and functional data for Seychelles caecilians. Better ecological data would potentially make it possible to unravel causal factors in the evolution and diversification of the radiation.

3.4.3 Taxonomic considerations

With the exception of two potentially newly discovered species (*H.* cf. *brevis* Praslin and *H.* cf. *brevis* CR) (see Chapters 4 and 6 for further details), the current taxonomy of Seychelles caecilians is supported in terms of numbers of species. *Hypogeophis* cf. *brevis* Praslin and *H.* cf. *brevis* CR, though not

completely independent in morphological analyses, differ significantly in character means (Table 1) and have key differences in characters that are useful in designating species elsewhere in Gymnophiona, namely the position of the tentacle and numbers of annular grooves. Intraspecific (mostly inter-island) variation notwithstanding, no compelling evidence was found for the presence of morphological cryptic species and although a full comparative inter-species analysis was not undertaken, key identification characters support the distinction of each species.

Some previous work has investigated intraspecific morphological variation in *H. rostratus* (Parker 1958; Taylor 1968, 1969) and these studies reported similar patterns to those recovered here: with northern (Parker 1958), Frégate (Taylor 1968), and southern (Parker 1958) lineages identified. These populations were previously described as independent subspecies (Parker 1958; Taylor 1968, 1969) but have received little subsequent recognition or attention. It may be useful to consider these subspecies as distinct evolutionary significant units for conservation management and it is suggested that they be implemented henceforth.

Specimens of *G. sechellensis* from Praslin are very similar morphologically to specimens from the rest of its known range. The species *G. diminutiva* Taylor, 1968, which was described from Praslin, has been synonymised with *G. sechellensis* (Wilkinson & Nussbaum 2006) and although the holotype of *G. diminutiva* was not examined in this study the results do not support any differences between the different island populations of *G. sechellensis.*

Based on morphological data only, the three populations recovered in the *H. brevis* group, along with the other five widely recognised species, should be recognised as distinct species units. The Seychelles caecilian fauna is considered to comprise eight morphologically distinct species.

CHAPTER 4

LIVING IN PARADISE: COMPARATIVE PHYLOGEOGRAPHY OF SEYCHELLES CAECILIAN AMPHIBIANS REVEALS DIFFERING EVOLUTIONARY HISTORIES

Abstract

Our understanding of the genetic patterns of intraspecific variation among Seychelles organisms is relatively lacking in comparison to other major island groups. Studies of comparative phylogeography on the Seychelles would provide insight into the patterns and processes that have affected the archipelagos biota. With this in mind this chapter rigorously investigates the phylogeography of the co-distributed caecilians of the Seychelles. The Seychelles caecilians provide an excellent opportunity to study patterns of comparative intraspecific variation because they are an ancient, morphologically distinct radiation with different life history strategies. Data was generated for one mitochondrial and four nuclear loci in 244 caecilian specimens. Contrasting patterns of intraspecific geographic variation was observed among the species: no geographic variation (*Grandisonia sechellensis* (in mtDNA) and *Praslinia cooperi*), a species with a basal northern- vs southern-island split (*Hypogeophis rostratus*), two species with a geographic split between specimens from the western island of Silhouette and elsewhere (*G. alternans* and *G. larvata*), and one species showing independence of each island population (*G. sechellensis* (in nuDNA). Based on our current knowledge of the ecologies and life histories of the Seychelles

caecilians it is unlikely that the observed genetic patterns of geographic variation are the result of a single causal factor.

4.1 Introduction

Determining the amplitude and geographic structure of genetic variation of organisms has become an important topic in modern evolutionary biology. Since its first use, phylogeography (Avise et al., 1987) has been growing rapidly across all major extant organismal groups, seeking to identify the extent to which past abiotic events have influenced current day diversity patterns. Phylogeographic studies have also had a major influence on systematics, especially through the identification of morphologically cryptic species (e.g. Rawlings et al., 2008; Sanguila et al., 2011; Vences et al., 2004).

Island faunas have received large amounts of attention in phylogeographic studies (e.g. Evans et al., 2003; Hawlitschek et al., 2012; Kuntner and Agnarsson, 2011a, 2011b; Rocha et al., 2013a; Valente et al., 2014; Wallace et al., 2009; Warren et al., 2003) but studies of island amphibian have remained comparatively scarce (e.g. Brown et al., 2010; Gehring et al., 2012; Maddock et al., 2014; Nussbaum and Wu, 1995; Sanguila et al., 2011; Stoelting et al., 2014; Taylor et al., 2012). This lack of attention is likely at least partly a result of the general rarity of amphibians occupying islands because the osmotic properties of their skin greatly limit the capacity for dispersal across saltwater barriers (Balinsky 1981; Duellman & Trueb 1986).

The Seychelles archipelago, an isolated group of islands in the Indian Ocean, has only in the last few years been the focus of detailed molecular

phylogeographic studies. Most of this work has been centred on several lizard groups*: Phelsuma* and *Urocotyledon* geckoes, and *Mabuya* and *Pamelaescincus* skinks (Rocha *et al.* 2010b; a, 2011, 2013a; Valente *et al.* 2014). Other, non-lizard organisms that have been studied include freshwater turtles (Silva *et al.* 2010a), frogs (Chapter 2; Maddock et al., 2014; Taylor et al., 2012), crabs (Daniels 2011)*,* and *Drosophila* flies (Legrand *et al.* 2009)

All of the phylogeographic studies of Seychelles lizards to date have found a general genetic split between populations on northern (including Praslin and La Digue) and southern (including Mahé and Silhouette) island groups (Rocha *et al.* 2010b; a, 2011, 2013a; Valente *et al.* 2014). Populations on the eastern island of Frégate were found to be genetically more similar to those of the southern island group for most taxa except in the case of the gecko *Phelsuma astriata* (in species tree analysis) (Rocha *et al.* 2013a) and the skink *Pamelaescincus gardineri* (Valente *et al.* 2014)*,* for which the Frégate populations of both species clustered with the northern island group.

The sooglossid frog *Sooglossus sechellensis* (Taylor *et al.* 2012) and freshwater crab *Seychellum alluaudi* (Daniels 2011) were found to have genetic splits between populations from northern islands, the western most island of Silhouette, and Mahé(+Frégate in *S. alluaudi*). Conversely, other organisms including *Drosphila* flies (Legrand et al., 2009, although see Legrand et al., 2011), freshwater turtles (Silva *et al.* 2010a) and treefrogs (Chapter 2; Maddock et al., 2014), show no clear geographic structuring in intraspecific variation despite occurring across multiple islands with known geographic breaks in other taxa.

Gymnophiona (caecilian amphibians) is a relatively little studied and perhaps the most poorly known order of extant vertebrates. Caecilians have a tropical (primarily Gondwanan) distribution occurring across Central and South America, Africa and Asia, and consist of approximately 200 recognised living species classified in ten families (Wilkinson *et al.* 2011; Kamei *et al.* 2012). All caecilians are limbless and the vast majority are fossorial, making them rarely encountered without dedicated field effort, which is likely one of the major causes for their relative biological neglect. Few investigations have been conducted looking at geographical patterns of genetic variation in caecilian amphibians. The most well studied species that has been studied to look for geographical patterns of variation is the São Tomé island dermophiid *Schistometopum thomense* (Nussbaum & Pfrender 1998; Stoelting *et al.* 2014). Stoelting et al. (2014) found high levels of geographic structuring of genetic variation matching high levels of morphological variation (Nussbaum & Pfrender 1998). High levels of intraspecific geographic variation have also been reported for the Chinese icthyophiid *Ichthyophis bannanicus* (Wang *et al.* 2015). In contrast, Gower et al. (2007) reported very little (and very weakly geographically-structured) genetic variation in the Indian ichthyophiid *I. bombayensis* along 1,500 km of its range.

The caecilians of the Seychelles might be considered a potentially insightful group to study comparative phylogeography because they consist of a single radiation (e.g. Frost et al., 2006; Pyron and Wiens, 2011; San Mauro et al., 2014, 2009; Wilkinson and Nussbaum, 2006) of largely co-distributed taxa occurring often in sympatry on between one and 11 of the granitic islands (Nussbaum, 1984; *pers. obs.*). The Seychelles caecilian radiation comprises

six nominal species plus two undescribed dwarf species (see Chapter 6: referred to in this chapter as *H.* cf. *brevis* CR, referring to its collection locality of Congo Rouge, Mahé, and *H.* cf. *brevis* Praslin from the island of Praslin), all of which are sampled in the study reported in this chapter. The Seychelles caecilians have been physically separated from their closest living relatives (Indian indotyphlids: San Mauro et al., 2014; Gower et al., 2011) since the separation of the Seychelles and Indian plates approximately 65MYA (e.g. Armitage et al., 2011; Collier et al., 2008; Gunnell et al., 2003).

The Gondwanan history of the Seychelles means that the seas between the granitic islands are relatively shallow (Davies & Francis 1964) meaning that the present-day islands would have experienced cyclical episodes of connection and separation in response to eustatic sea-level fluctuations (Colonna *et al.* 1996; Rohling *et al.* 1998; Siddall *et al.* 2003; Camoin *et al.* 2004; Miller *et al.* 2005). The westernmost granitic island of Silhouette is currently separated from the remaining main granitic islands by a deeper (~60 m) trench in the ocean floor and compared to other islands (average depth of ~40 m) (Rocha *et al.* 2013b). These fluctuations in global sea levels have been implicated in causing at least some of the major geographic structure observed in genetic variation in some Seychelles organisms.

Despite their monophyly and partly sympatric occurrence, life histories vary extensively within Seychelles caecilians. As far as is known, all species are oviparous (R.A. Nussbaum unpublished; pers. obs.) but *Hypogeophis rostratus* has no larval stage, *Grandisonia larvata* and *G. sechellensis* have a brief larval stage, and *Praslinia cooperi* has one of the most extensive, aquatic

larval stages of any caecilian globally, (Nussbaum, 1984; San Mauro et al., 2014). The early life-history stages of *G. alternans* and the *H. brevis* group are unknown. The disparity in life history modes (and possibly other aspects of ecological niches) might explain how relatively many species co-occur in sympatry on the Seychelles.

This study is the largest multitaxon investigation thus far of geographic patterns of genetic variation across the Seychelles. All known Seychelles caecilian species are sampled and analysed for variation in mitochondrial and nuclear genetic markers. Sample coverage across the ranges of all caecilian species is high at the island level, with only one island population for each of *H. rostratus, G. alternans* and *G. larvata* unsampled out of a maximum of 11, 6 and 6 islands, respectively.

4.1.1 Hypotheses

An accusation that can be levelled at some interpretations in some phylogeographic studies is that they (are at least presented as) post hoc narratives. Given that it is more powerful to test a priori formulated hypotheses, the following predictions are put forward:

A. All Seychelles caecilian species occurring on both northern and southern islands will show a north vs. south island split in genetic variation, in common with what has been observed in lizards (Rocha *et al.* 2010b; a, 2011, 2013a; Valente *et al.* 2014). Molecular and morphological variation in *H. rostratus* has already been found to be

partitioned into northern and southern (and eastern) island groups (Parker, 1958; Taylor, 1969, 1968; Nussbaum et al., unpublished).

B. All Seychelles caecilian species will show high levels of spatially structured genetic variation among and possibly even within islands. Support for this prediction comes from substantial intra-island variation in the São Tomé island caecilian *S. thomense* (Nussbaum & Pfrender 1998; Stoelting *et al.* 2014) and evidence for substantial genetic variation in Seychelles organisms, including among populations of other taxa that are presumably less vagile than lizards (Daniels 2011; Taylor *et al.* 2012).

C. In contrast to B, it might be argued that because all Seychelles caecilian species (except *H. brevis* sensu stricto) occur on multiple islands they might be good dispersers and therefore for alleles to be expected to have been relatively frequently exchanged among populations, leading to relatively little geographic structuring of genetic variation. This hypothesis would result in a 'pattern' similar to that observed within the Seychelles treefrog *Tachycnemis seychellensis* (Chapter 2; Maddock et al., 2014)

D. Co-distributed Seychelles caecilian species will share the same patterns of spatial genetic variation. In other Seychelles organisms that share broadly similar ecologies and life-history strategies, such as the lizards (Rocha *et al.* 2010b; a, 2011, 2013a; Valente *et al.* 2014), a common phylogeographic pattern has been observed.

E. In contrast to D, given their distributional, morphological and life-history diversity, co-distributed Seychelles caecilians will have different spatial

patterns of genetic variation. Ecological (habitat) specialists occurring on multiple islands (e.g., *P. cooperi,* restricted to higher altitudes on only the two highest islands) will show greater inter-island genetic variation than more generalist species (e.g., *H. rostratus*, found at many altitudes across 11 islands) based on the assumption that size of distribution is a proxy for relative ecological specialisation and/or dispersal ability.

4.2 Methods

Seychelles caecilian samples were collected between 1989 – 1991 (University of Michigan Museum of Zoology) and 2013 – 2015 (collected during the course of this PhD) from across their ranges (Fig. 1). Tissue samples consisted variably of muscle, heart, liver and non-lethal sampling (Maddock et al., 2014). A total of 244 samples were used in this chapter, covering all species across their known ranges (see Table 1.1) except for *Hypogeophis rostratus* where no samples were available from the island of Grand Soeur, and for *Grandisonia alternans* and *G. larvata* where no samples were available from the island of Félicité.

The largest island of Mahé is treated as three distinct units in this chapter: northern-Mahé, mid-Mahé and southern-Mahé. Northern-Mahé refers to specimens occurring north of between 4°39'28.65"S 55°24'35.74"E and 4°37'9.53"S 55°27'19.15"E, southern-Mahé refers to specimens occurring south of between 4°41'58.28"S 55°27'40.63"E and 4°40'58.85"S 55°31'56.68"E, and specimens from mid-Mahé occur between specimens from northern- and southern-Mahé. The separation between northern- and mid-

Mahé is approximately the area where the highest peaks in the Morne Seychellois National Park (the highest in the Seychelles) occur, potentially acting as a natural barrier to dispersal. The separation between mid- and southern-Mahé is situated in an area where there is a large sampling gap between specimens.

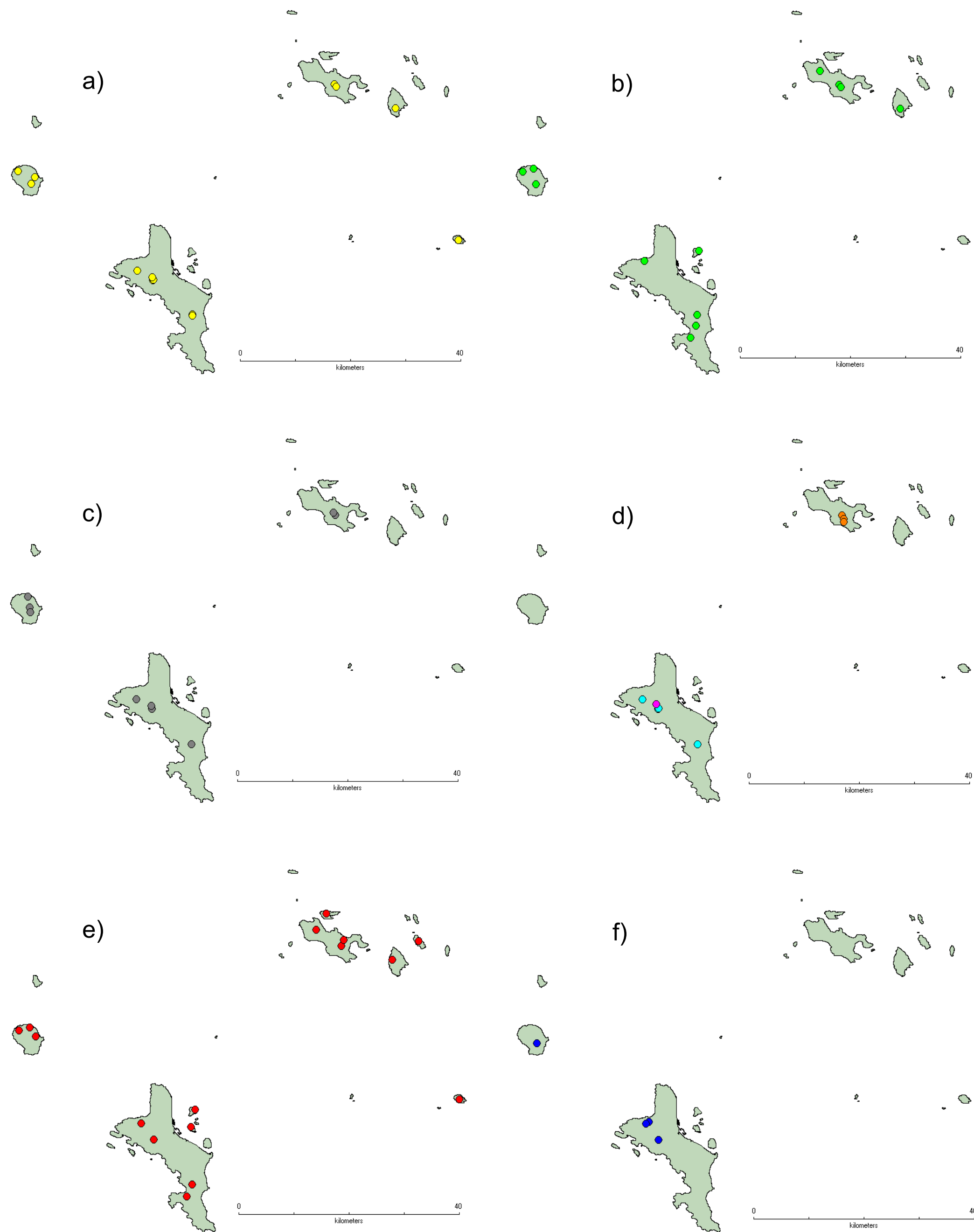

Fig 1. Maps of the granitic Seychelles showing molecular sampling localities of each focal species of caecilian: a) *Grandisonia alternans*, b) *G. larvata*, c) *G. sechellensis*, d) *Hypogeophis brevis (H. brevis sensu* strcito = turquoise*, H*. cf. brevis CR = pink, *H*. cf. brevis Praslin = orange) e) *H. rostratus*, f) *Praslinia cooperi*. Points used on each map are the same colours used throughout this chapter

4.2.1 Laboratory protocols, sequence editing and alignment

Complete genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit. Qiagen kit extraction followed manufacturer guidelines with the exception of the final suspension of the extracted DNA, which was suspended twice in100µl buffer AE.

Standard PCR was used to amplify DNA fragments from one mitochondrial locus: cytochrome *b* (*cytb*); and four nuclear loci: pro-opiomelanocortin (*pomc*) and brain-derived neurotrophic factor (*bdnf*), and the two anonymous nuclear markers *brev5* and *rost5* (Lewis *et al.* 2014). See Table 1 for primer information. PCR reaction volume was 25µl and consisted of 12.5µl of MyTaq Mix x 2, 9.5µl of ddH_2O, 1µl of forward and reverse primers, and 1µl of template DNA. Cycling conditions for ANL followed (Lewis *et al.* 2014). All other amplification conditions were as follows: 2:00 at 95°C; 40 x [0:30 at 95°C; 0:30 at 48°C for *cytb* and 58°C for *bdnf* and *pomc*; 0:30°C at 72°C]; and a final extension of 72°C for 4:00.

Of the 244 samples in the *cytb* dataset a subset of 162 were used for the nuclear dataset. These 162 samples were selected across the ranges of each species and covered all major haplogroups identified from the *cytb* data. The sequence of the Indian indotyphlid *Gegeneophis seshachari* BNHS4231 from GenBank (HQ444101) was used as an outgroup for phylogenetic analyses of the *cytb* dataset based on Indian indotyphlids being a monophyletic sister group to the Seychelles radiation (e.g. San Mauro et al., 2004).

Sequences were proof read and edited in Geneious v.6.1.4 (Biomatters). Cleaned sequences were aligned using MUSCLE (Edgar 2004a) in Geneious and checked for unexpected indels and stop codons in MEGA v.6.0.6 (Tamura

et al. 2013). Mean inter- and intraspecific *p*-distances were estimated using these alignments in MEGA.

Table 1 Primers used in this chapter for PCR and sequencing.

Locus	Primer name	Primer sequence (5' – 3')	Reference
cytb	L14724	CGAAGCTTGATATGAAAAACCATCGTTG	Irwin et al. (1991)
	CB3H	GGCAAATAGGAAGTATCATTCTG	Moritz et al. (1992)
pomc	POMC_DRV_F1	ATATGTCATGASCCAYTTYCGCTGGAA	Vieites et al. (2007)
	POMV_DRV_R1	GGCRTTYTTGAAWAGAGTCATTAGWGG	Vieites et al. (2007)
bdnf	BDNF_DRV_F1	ACCATCCTTTTCCTKACTATGG	Vieites et al. (2007)
	BDNF_DRV_R1	CTATCTTCCCCTTTTAATGGTC	Vieites et al. (2007)
brev5	brev5_F	CATCAGGTCATTGGCGTTTA	Lewis et al. (2014)
	brev5_R	GAGTGCAGGGACCAAATACC	Lewis et al. (2014)
rost5	rost5_F	TGTCAACTGCCCTCTGTGTC	Lewis et al. (2014)
	rost5_R	AAATTCACAGGCCAAACAGG	Lewis et al. (2014)

4.2.2 Mitochondrial variation

Maximum likelihood (ML) and Bayesian Inference (BI) analyses were carried out on the *cytb* data. RaxML v.8.0.24 (Stamatakis 2014) and MrBayes v.3.2.2 (Ronquist *et al.* 2012) were used to reconstruct ML and BI phylogenies, respectively. Partitioning strategy and best-fit models of nucleotide evolution were selected based on results from PartitionFinder v.1.1.1 (Lanfear et al., 2012) with the potential for three partitions to be selected based on codon positions. MrBayes was run for 10^6 generations and sampled every 10,000 generations with one cold and three heated chains. Chains were checked for convergence using Tracer v1.5 (Drummond & Rambaut 2007) and the first 10% of trees were discarded as burn-in. Five hundred bootstrap iterations

were employed for the RaxML analyses using the default option (Stamatakis 2006) of the GTR+CAT substitution model for each partition followed by GTR+G inference for the final tree topology.

To investigate if common geographic splits in genetic variation occurred during the same time period (i.e., were potentially caused by the same abiotic event), relative dating (Loader *et al.* 2007) was performed in BEAST v2.1.3 (Bouckaert *et al.* 2014). An arbitrary calibration of 10 was placed on the ancestor of all Seychelles caecilians under a normal distribution using a strict clock following recommendations from preliminary runs. BEAST was run for 40^6 generations and sampled every 10,000 generations. Convergence was assessed using Tracer v1.5 (Drummond & Rambaut 2007).

4.2.3 Nuclear variation

To determine whether heterozygous positions were genuine, the nuclear loci were run through PHASE v.2.1.1 (Stephens *et al.* 2001; Stephens & Scheet 2005) three times each with a random starting seed selected using random.org. Files for PHASE were prepared using seqPHASE (Flot 2010). Heterozygous positions were accepted as true if they received a score >0.7 based on findings by Harrigan *et al.* (2008); these files were used in all subsequent analyses.

Haplotype networks were constructed using NETWORK v.4.611 (fluxus-engineering.com) using the median joining algorithm. NETWORK treats insertions and deletions (indels) as missing data despite them often seemingly carrying phylogenetic information. To ensure that indels were used in the analyses gaps were coded as an additional (fifth) base that was not present in

that position of the alignment; if there was more than a single consecutive gap present then it was deemed to be the same insertion (and no variation was seen across all samples over that portion of the sequence) then bases were removed so that it was only considered as a single mutation event in the analyses, i.e. one base pair. All-species data sets for each locus were analysed to inspect for allele sharing among nominal species. Intra-specific patterns of variation were also assessed for each locus for each species.

Combining patterns of genetic distances for different loci can help to determine common patterns of genetic structure. POFAD v.1.03 (Joly & Bruneau 2006) was used to conduct combined analyses for each species using the four nuclear markers. Using genetic distance data from individual loci POFAD creates a data matrix that gives each locus equal waiting, thus preventing faster evolving loci from disproportionately influencing the results. Genetic distance data were exported from MEGA as *p*-distances. MEGA cannot incorporate raw indels, so the same approach described above for NETWORK analyses was used to include them. The equally weighted results were visualised in a PCA plot, which was run through MVSP (www.kovcomp.com). The NeighborNet algorithm (Bryant & Moulton 2004) was also used to construct a distance network of the equally weighted distance matrix using SplitsTree v.4.13.1 (Huson & Bryant 2006).

The standardised genetic distances for the nuclear dataset were analysed using an isolation by distance Mantel test model (Mantel 1967) using the Isolation by Distance Web Service (IBDWS) (Jensen *et al.* 2005). IBDWS was run for 10,000 randomisations for each multi-locality species. The distance matrix for the genetic distances was created using the Geographic

Distance Matrix Generator v.1.2.3 (Ersts; http://biodiversityinformatics.amnh.org/open_source/gdmg/).

Table 2 Summary information for each of the five loci analysed in this chapter. N = number of samples; Bp = number of base pairs; VI = Variable sites; PI = Parsimony informative sites; D = Tajima *D*; π = Nucleotide diversity.

Locus	N	Bp	No. Indels	VI	PI	D	π
cytb	244	809	-	328	295	2.791301	0.126008
bdnf	152	713	-	32	22	-0.595952	0.005585
pomc	142	509	-	80	71	-0.782259	0.018625
brev5	156	298	7	80	72	-0.963722	0.028852
rost5	141	304	5	49	44	-0.208930	0.024206

Table 3 Within species *p*-distance means for all eight species for each of the five loci.

Species	*cytb*	*bdnf*	*brev5*	*pomc*	*rost5*
G. alternans	0.042	0.002	0.007	0.005	0.003
G. larvata	0.030	0.001	0.005	0.004	0.006
G. sechellensis	0.007	0.001	0.003	0.003	0.007
H. brevis	0.012	0.000	0.009	0.004	0.008
H. rostratus	0.022	0.001	0.008	0.004	0.004
P. cooperi	0.002	0.000	0.034	0.001	0.004
H. cf. *brevis* Praslin	0.004	0.002	0.003	0.001	0.002
H. cf. *brevis* CR	0.011	0.000	0.008	0.002	-

4.3 Results

4.3.1 Mitochondrial variation

Intraspecific sequence diversity and mean *p*-distances vary considerably among species (Tables 2 and 3). All eight sampled species are strongly monophyletic (Fig. 2). Three (*Grandisonia alternans, G. larvata* and *Hypogeophis rostratus*) of the sampled species had notably geographically

structured intraspecific haplotype variation. In contrast, *Praslinia cooperi, G sechellensis, H. brevis* and the two undescribed dwarf species showed no notable geographic structuring of *cytb* variation. *Grandisonia alternans* and *G. larvata* share a similar geographic pattern of mitochondrial variation (but see below) when compared to that observed within *H. rostratus*. The former pair have a basal split between specimens from the southwestern island of Silhouette and the remaining islands, whereas *H. rostratus* shows a northern-southern island basal split.

For *H. rostratus* there is a maximally supported northern+eastern island clade including all samples from the islands of Praslin, La Digue, Curieuse, Félicité and Frégate. Support for splits within this clade are generally low but a well-supported group is present consisting of all populations from Praslin, Curieuse and Frégate. The populations from the southern islands (Mahé, Silhouette, Cerf, St Anne) are together not compellingly monophyletic (but see relative dating analysis results, in which the southern island specimens are monophyletic). Only a subset of Mahé samples (SM181, SM182, SM358, SM359, SM361, SM363, SM364) and three Silhouette samples (RAN31410, SM209, SM232) form well-supported monophyletic within-island groups.

For *cytb G. larvata* has a deep (mean *p*-distance = 0.0501), maximally supported basal split between populations from the southwestern island of Silhouette versus the remaining islands that this species occurs on. Excluding Silhouette, populations from the southern islands of Mahé and Ste Anne form the sister clade to populations on Praslin and La Digue in the north of their range (mean *p*-distance = 0.0133).

The basal split within *G. alternans* is also deep (mean *p*-distance = 0.0755) and maximally supported. One half of the split, with the largest amount of sub-structuring, comprises a clade of all specimens from the southwestern island of Silhouette as sister (mean *p*-distance = 0.0431) to a clade comprising all of the most northerly (and generally higher-altitude) specimens from Mahé. The other half of the basal split comprises three subclades - all the specimens from the rest of Mahé, the eastern island of Frégate, and the northern islands of Praslin and La Digue, though the relationships among these three subclades is not resolved. Within the northern-island group specimens from La Digue are monophyletic. A single specimen (SM131) from Praslin forms the sister taxon to the population on La Digue thus rendering populations from Praslin paraphyletic. Specimens SM343 and SM352 from the second major group were collected on the slopes of Congo Rouge, Mahé within the relative proximity of specimen SM350 from the first major clade. Henceforth, in nuDNA analyses the Mahé specimens in the first major group are considered a northern-Mahé group despite occurring in the mid-Mahé geographic region (see section 4.2 for details) and geographically close specimens e.g. SM343 and SM352 are considered members of a mid-Mahé group.

Hypogeophis brevis shows low levels of geographic structuring. The single specimen from the most northerly sampled locality of Mare Aux Cochons (SM193) being a somewhat distinct (mean *p*-distance = 0.0278) sister to the remaining populations. Among the remaining populations, the most southerly sampled population from La Reserve (southern-Mahé) is a distinct (mean *p*-distance = 0.0130) sister to a clade comprising all Morne

Seychellois specimens (mid-Mahé) (except the single individual from Mare aux Cochons (northern-Mahé)).

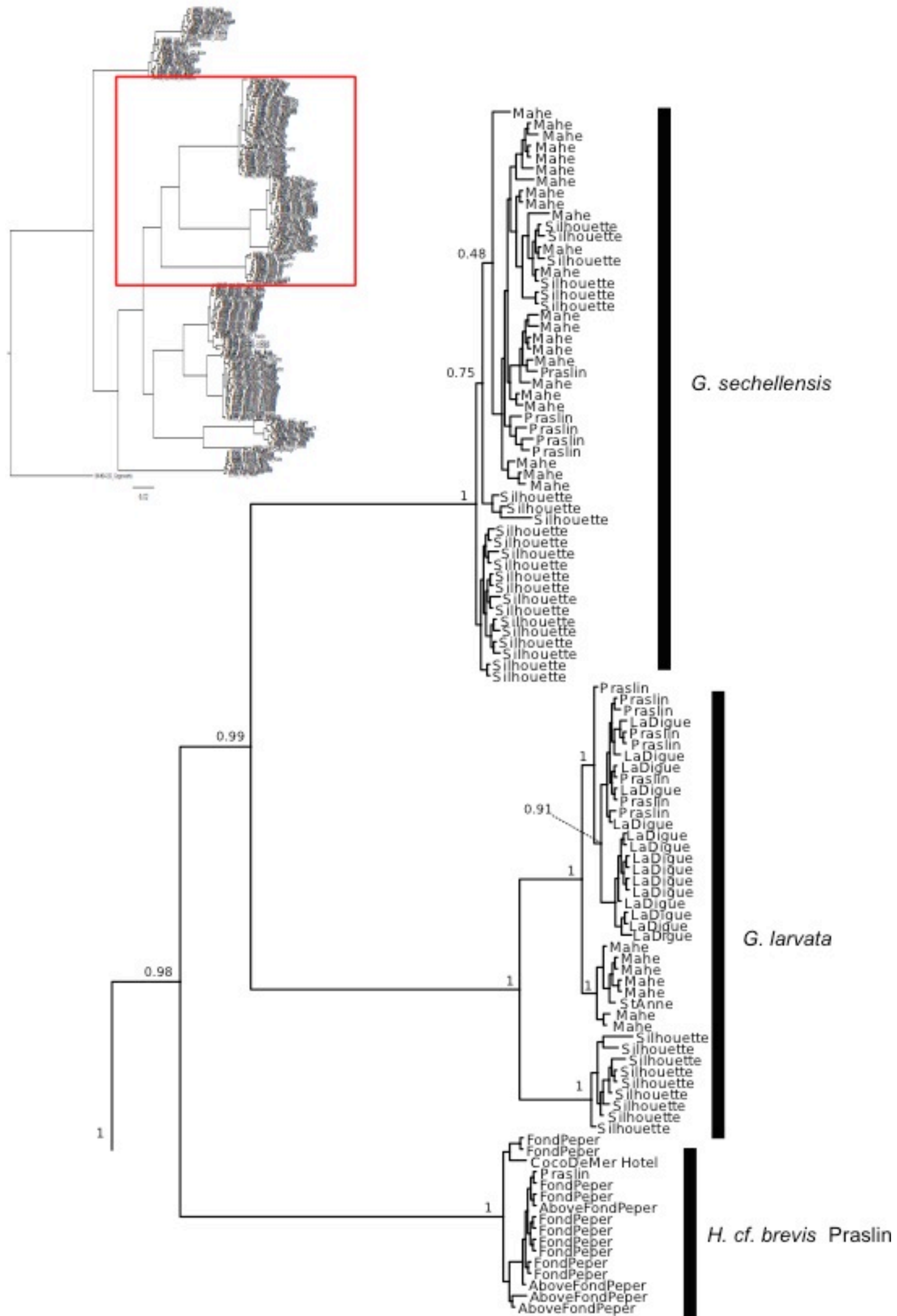

Fig 2a. Bayesian inference tree of mtDNA (*cytb*) sequences for all Seychelles caecilian species analysed with mrBayes. Numbers on branches are Bayesian posterior probabilities. Scale bar indicates number of expected mutations per site.

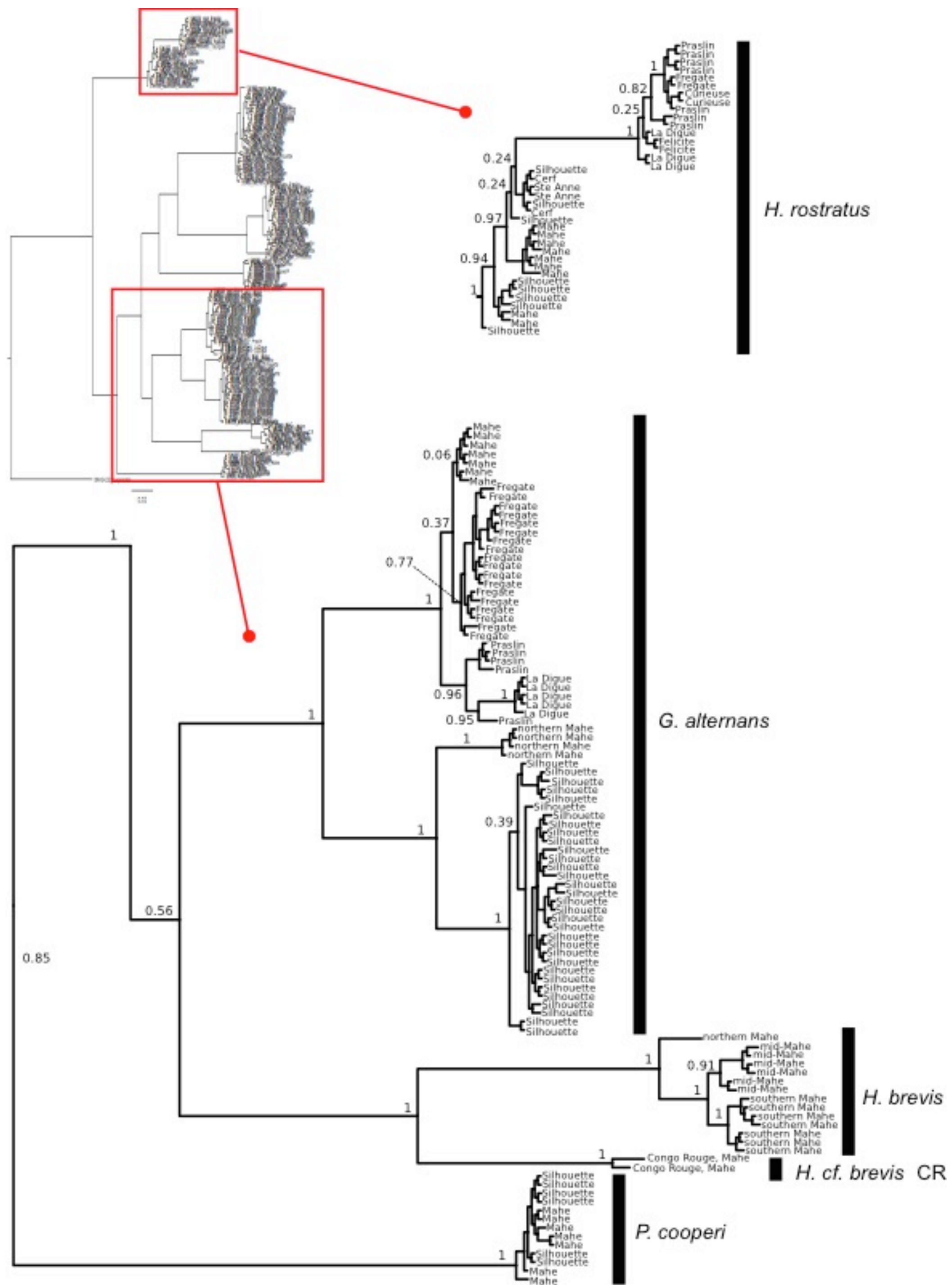

Fig 2b. Bayesian inference tree of mtDNA (*cytb*) sequences for all Seychelles caecilian species analysed with mrBayes. Numbers on branches are Bayesian posterior probabilities. Scale bar indicates number of expected mutations per site.

4.3.2 Relative dating

The mitochondrial relative dating analysis (Fig. 3) was unable to reject the possibility that all but one of the geographically structured basal intraspecific splits in Seychelles caecilians occurred at the same time. The exception is the older basal divergence within *G. alternans* between a Silhouette + northern Mahé clade versus a clade of other populations. The only other divergence potentially contemporaneous with the primary split in *G. alternans* is the interspecific split between *H. brevis* and *H.* cf. *brevis* CR. The two intraspecific splits between populations from Silhouette and elsewhere (in *G. alternans* and *G. larvata*) have strongly overlapping relative dating estimates.

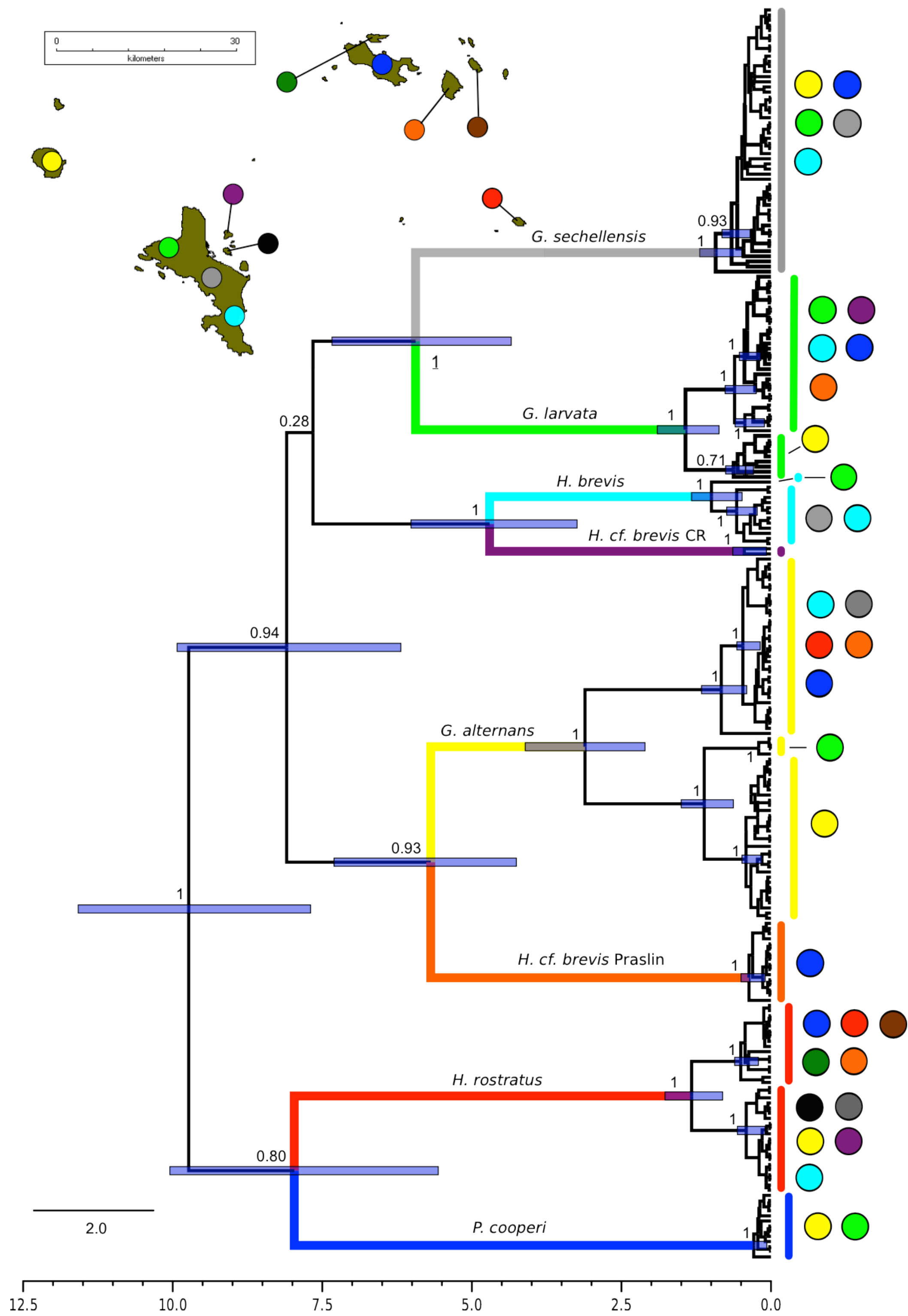

Fig 3. Bayesian inference relative dating tree inferred from mtDNA (*cytb*) sequences using BEAST. Coloured branches and bars refer to the colours used in Figs. 1, 4. Coloured circles refer to geographic localities represented in clade based on the map and used in Figs 5-12. Scale bar below is an arbitrary timeframe.

4.3.3 Nuclear variation

4.3.3.1 Interspecific variation

For *bdnf* there is some allele sharing between species. The commonest haplotype occurs mostly in the sampled *Hypogeophis rostratus* but is also shared with specimens of *Grandisonia alternans* (a single allele from RAN31314 (middle Mahé), SM323 (La Digue), SM329 (La Digue) and SM480 (Praslin), and both alleles from SM303 (Praslin)) and with a specimen of *G. larvata* (both alleles from SM361 (southern Mahé)). All other species are characterised by unique haplotypes only. All species have intraspecific genetic diversity (number of unique haplotypes: *G. alternans* = 7, *G. larvata* = 2, *G. sechellensis* = 6, *H. brevis* = 2, *H. rostratus* = 5, and *H.* cf. *brevis* Praslin = 4) except for *Praslinia cooperi* and *H.* cf. *brevis* CR. Fig. 4).

The *brev5* haplotype network (Fig. 4b) shows that all except one haplotype is unique among species for this marker. The shared haplotype occurs predominantly in *G. larvata* but a single specimen of *P. cooperi* (RAN31310) shares both alleles with it. All species contain multiple independent haplotypes (number of unique haplotypes: *G. alternans* = 16, *G. larvata* = 6, *G. sechellensis* = 8, *H. brevis* = 6, *H. rostratus* = 8, *P. cooperi* = 8, and *H.* cf. *brevis* CR = 3) except for *H.* cf. *brevis* Praslin, which only has one haplotype. A single specimen of *G. larvata* (RAN31190)*, P. cooperi* (RAN31322) and one allele of *H.* cf. *brevis* CR (SM284) are not most similar to conspecifics.

For the *pomc* data all species show independence from conspecifics except for two alleles shared between *G. larvata* and *G. sechellensis*. The two haplotypes that are shared are represented by a single allele from each

species. All species display multiple independent haplotypes (unique haplotypes: *G. alternans* = 26, *G. larvata* = 17, *G. sechellensis* = 20, *H. brevis* = 15, *H. rostratus* = 21, *P. cooperi* = 6, *H.* cf. *brevis* CR = 4, and *H.* cf. *brevis* Praslin = 7).

In contrast to the other loci examined there is no interspecific allele sharing for *rost5*, for which all species have multiple haplotypes (*G. alternans* = 6, *G. larvata* = 9, *G. sechellensis* = 13, *H. brevis* = 9, *H. rostratus* = 8, *P. cooperi* = 5, and *H.* cf. *brevis* Praslin = 4). Unfortunately, *rost5* could not be amplified for either of the sampled specimens of *H.* cf. *brevis* CR.

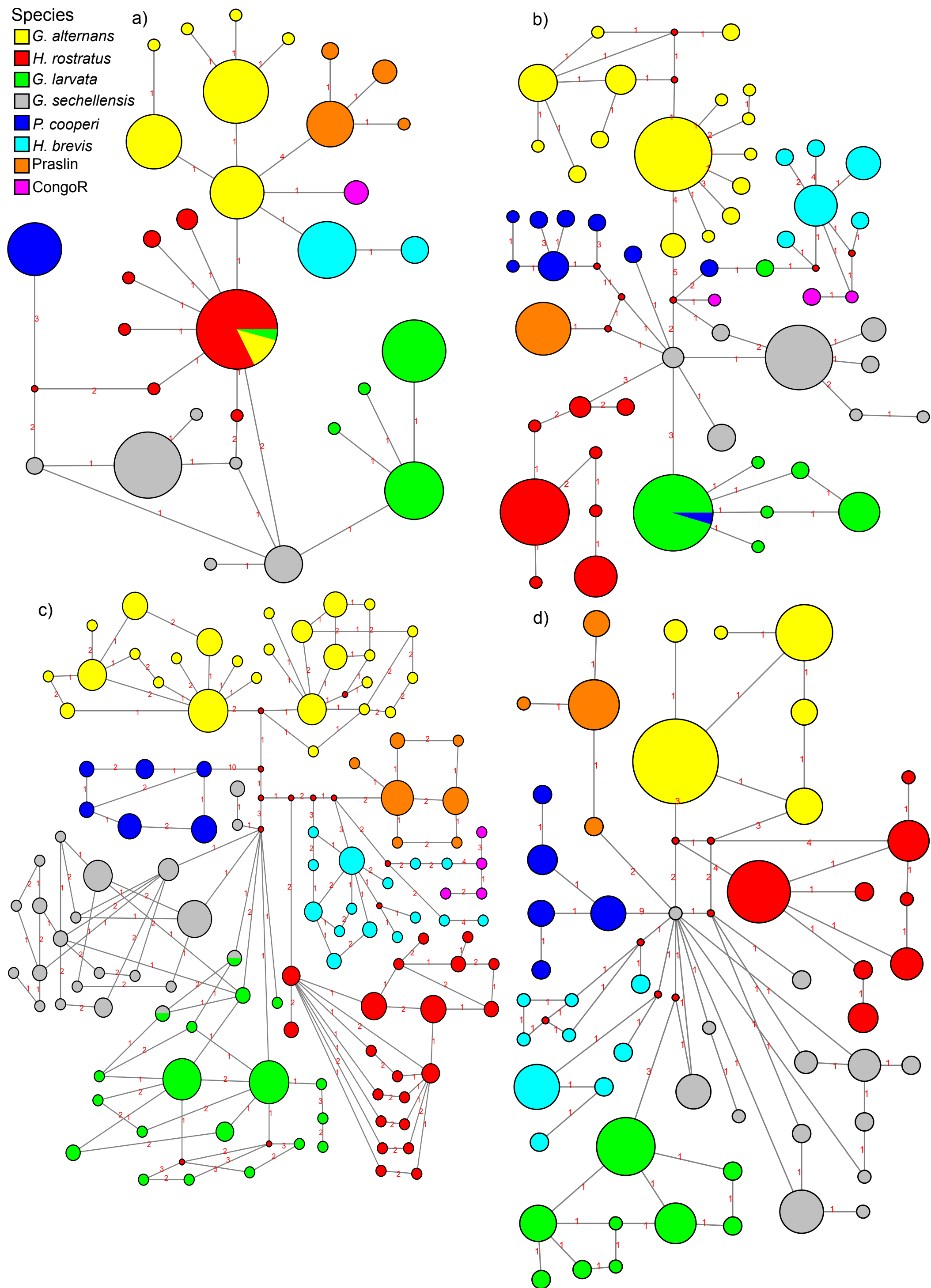

Fig 4. Median-joining interspecific haplotype networks for the Seychelles caecilians in four nuclear loci: a) *bdnf*, b) *brev5*, c) *pomc*, d) *rost5*. Colours in the key refer to species and are used to represent the species in each haplotype network. In the key "PRASLIN" refers to *H.* cf. *brevis* Praslin, and CONGOR refers to *H.* cf. *brevis* CR.

4.3.3.2 Intraspecific variation – Grandisonia alternans

In all haplotype networks for the nuclear loci (Fig. 5) *G. alternans* specimens from Silhouette have at least two unique haplotypes not shared with specimens from other islands. However, there is small amount of sharing of alleles with other populations for all loci. Specimens from Frégate, Praslin and southern Mahé have at least one unique haplotype for *pomc* and *brev5*. For *pomc* all specimens sampled from Frégate have haplotypes not found on any other island. Specimens from northern Mahé are generally more similar to specimens from Silhouette except that this is not as clear-cut for *pomc* and *rost5*, for which Silhouette haplotypes are more similar to those from southern Mahé or more geographically widespread haplotypes. However, for all loci specimens from northern Mahé share at least one allele with specimens from Silhouette and from the rest of Mahé. The mid-Mahé specimens include a single specimen (SM343) that shares a single allele with specimens from Silhouette and northern Mahé. The mid-Mahé SM343 is from the same locality as specimen SM350, which groups with northern Mahé specimens in mtDNA analyses but not in nuDNA. The independent haplotype shared between mid- and northern Mahé specimens in *brev5* is restricted to the same two specimens (SM343, SM350). The mid-Mahé specimen in the mid- northern Mahé haplotype group is representative of a single allele from specimen SM343.

The standardised *p*-distance analyses for the combined nuclear dataset (Figs. 11, 12) support the distinctiveness of specimens from Silhouette, and the close similarity between specimens from northern Mahé and Silhouette.

Specimens from Frégate comprise a cluster but specimens from other single islands are more disparate and do not cluster together.

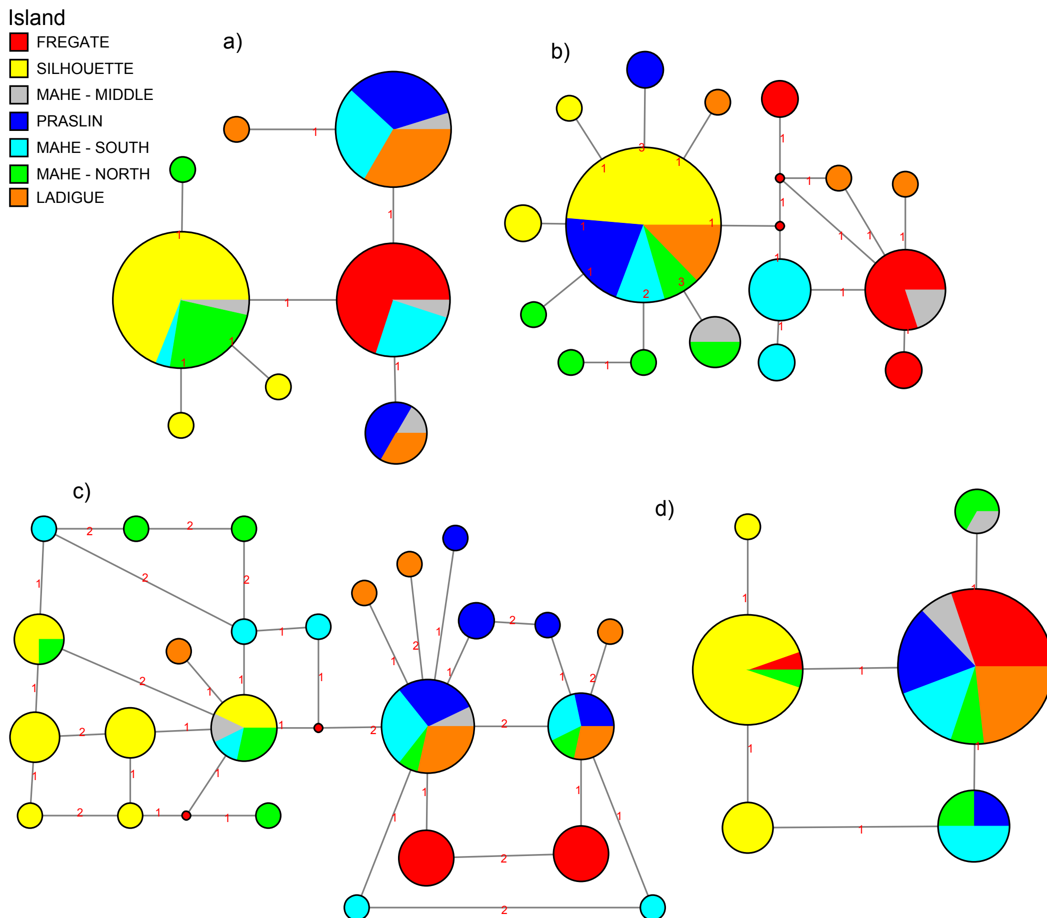

Fig 5. Median-joining haplotype networks for *Grandisonia alternans* in four nuclear loci: a) *bdnf*, b) *brev5*, c) *pomc*, d) *rost5.* Colours in the key refer to locality (as in Fig. 3) origin of each specimen.

4.3.3.3 Intraspecific variation – Grandisonia larvata

For all nuclear loci *G. larvata* populations from Silhouette have at least one haplotype not shared with populations from other islands (Fig. 6). For all loci except *bdnf,* all *G. larvata* from La Digue have at least one haplotype not shared with specimens from other islands. For *pomc* and *brev5* specimens from northern Mahé have at least one unique haplotype, and for *pomc* and

rost5 specimens from Praslin have single unique haplotypes. For *pomc* all island groups contain unique haplotypes. Mahé specimens share no alleles with each other for *rost5*.

Standardised *p*-distance analyses for the full nuclear dataset (Figs. 11, 12) mostly support a grouping of Silhouette samples. One exception is specimen SM231, which is probably explained by sequence data being available only for *bdnf* for this specimen. All nuclear loci were amplified for Silhouette specimen SM240 which is more similar to specimens from Praslin. The specimen (RAN31598) from Ste Anne is most similar to populations on Mahé.

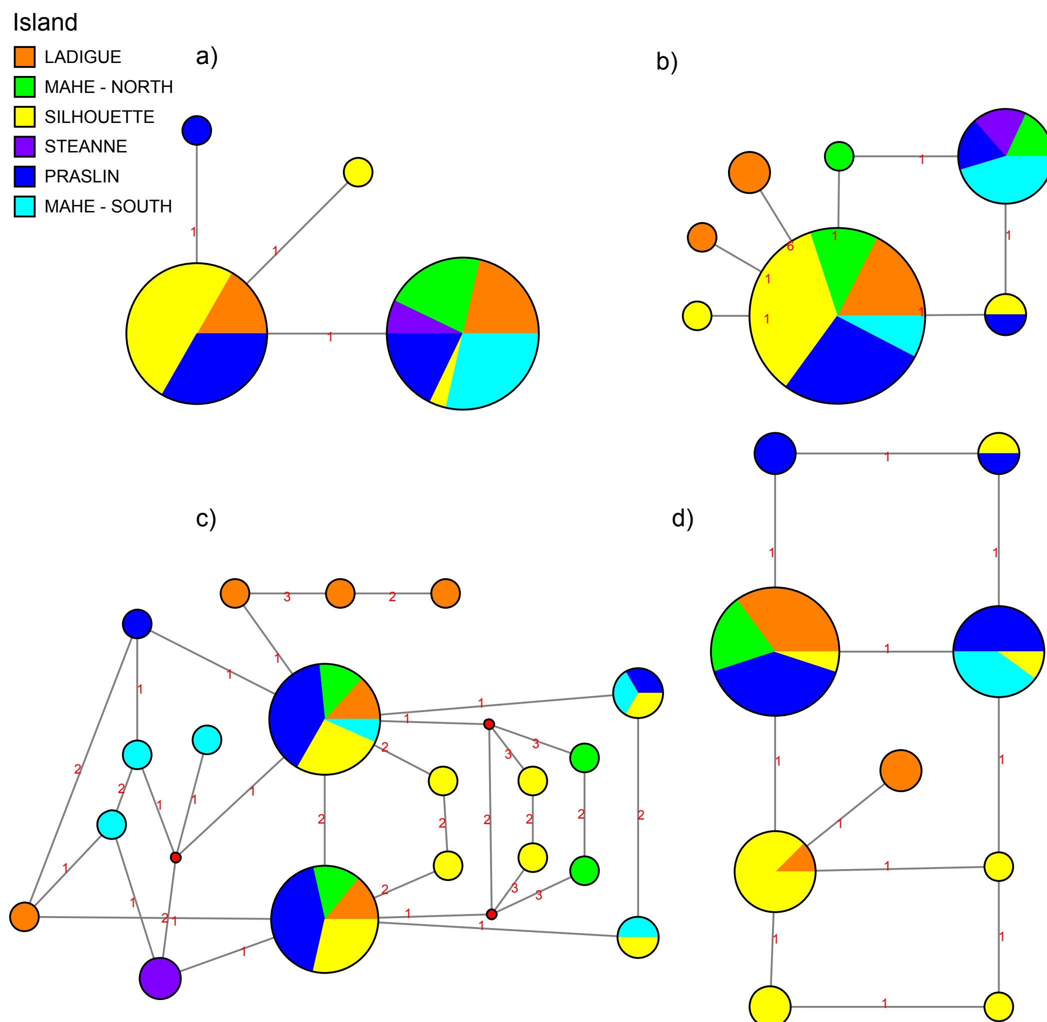

Fig 6. Median-joining haplotype networks for *Grandisonia larvata* in four nuclear loci: a) *bdnf*, b) *brev5*, c) *pomc*, d) *rost5.* Colours in the key refer to locality (as in Fig. 3) origin of each specimen.

4.3.3.4 Intraspecific variation – Grandisonia sechellensis

All sampled nuclear markers for *G. sechellensis* display independent haplotypes for specimens sampled from populations from Silhouette and mid-Mahé (Fig. 7). For all loci except *bdnf* individuals from southern Mahé have at least one independent haplotype not shared with populations from other islands, and for *bdnf* and *pomc* specimens from Praslin have at least one independent haplotype not shared with other populations.

Analysis of the combined nuclear standardised *p*-distances (Figs. 11, 12) supports the distinctiveness of specimens from the northern island of Praslin versus the remaining populations, and also of specimens from Silhouette (with the inclusion of specimen RAN31390 from Mahé) occupying an intermediate position between specimens from Mahé. Specimens SM371 (southern Mahé), SM383 (mid-Mahé), SM478 (Praslin) and RAN31249 (Silhouette) do not cluster closely with conspecifics but all of these lack sequence data for at least one locus.

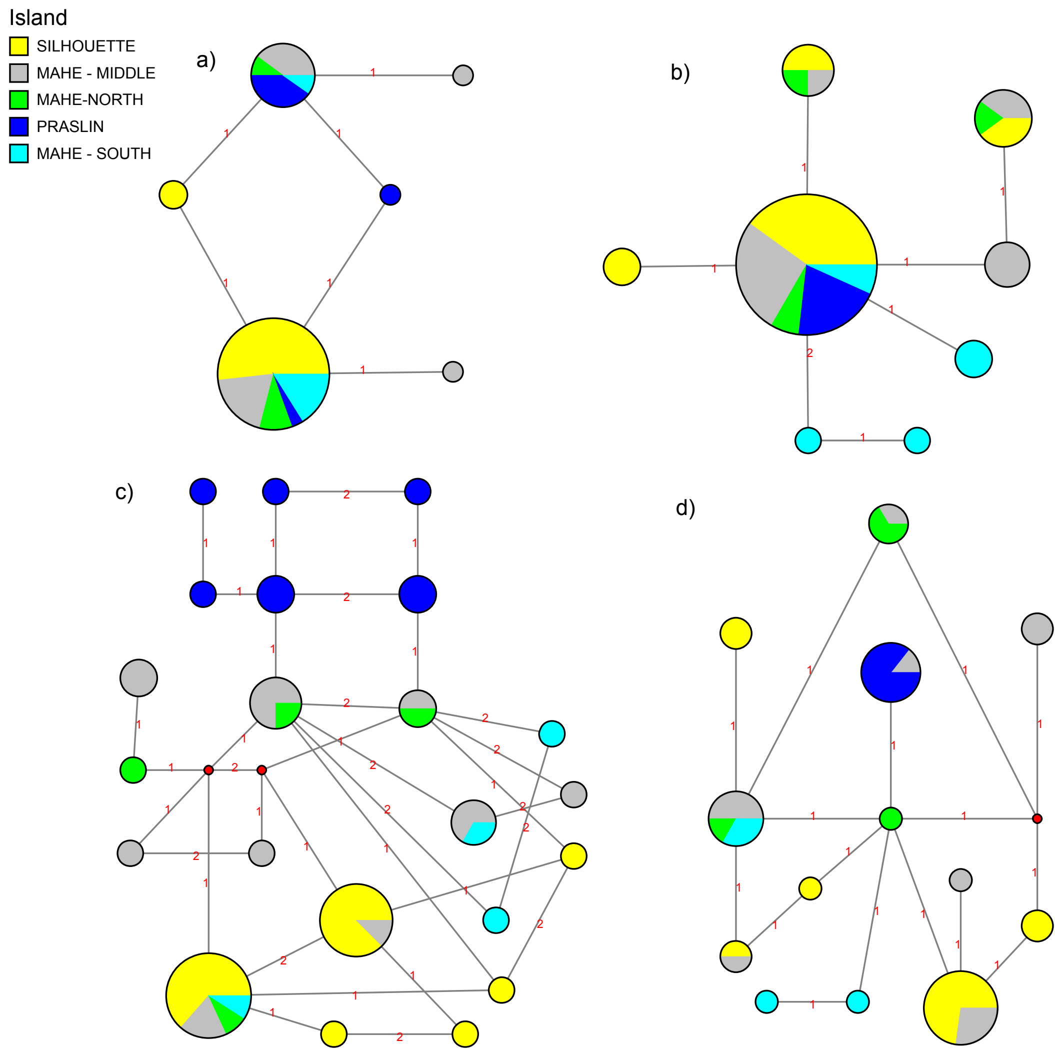

Fig 7. Median-joining haplotype networks for *Grandisonia sechellensis* in four nuclear loci: a) *bdnf*, b) *brev5*, c) *pomc*, d) *rost5*. Colours in the key refer to locality (as in Fig. 3) origin of each specimen.

4.3.3.5 Intraspecific variation – Hypogeophis brevis

In all haplotype networks for *H. brevis* except *bdnf* (haplotype network not shown because it includes only two haplotypes) the single northernmost locality specimen SM193 is separated by at least two mutational steps from conspecifics (Fig. 8). For the three loci for which networks are shown (*pomc, brev5, rost5*) specimens from mid- and southern Mahé share at least one haplotype with each other but also have independent haplotypes. For *pomc* specimen SM193 is closer to haplotypes from mid- Mahé whereas in rost5 it shares a closer affinity to southern Mahé haplotypes.

The standardised *p*-distance analyses for the combined nuclear dataset (Figs. 11, 12) clearly support the distinctiveness of the northernmost Mahé specimen SM193. The other specimens do not show clear geographically structured variation although specimens from the southern end of the sampled range are somewhat more clustered.

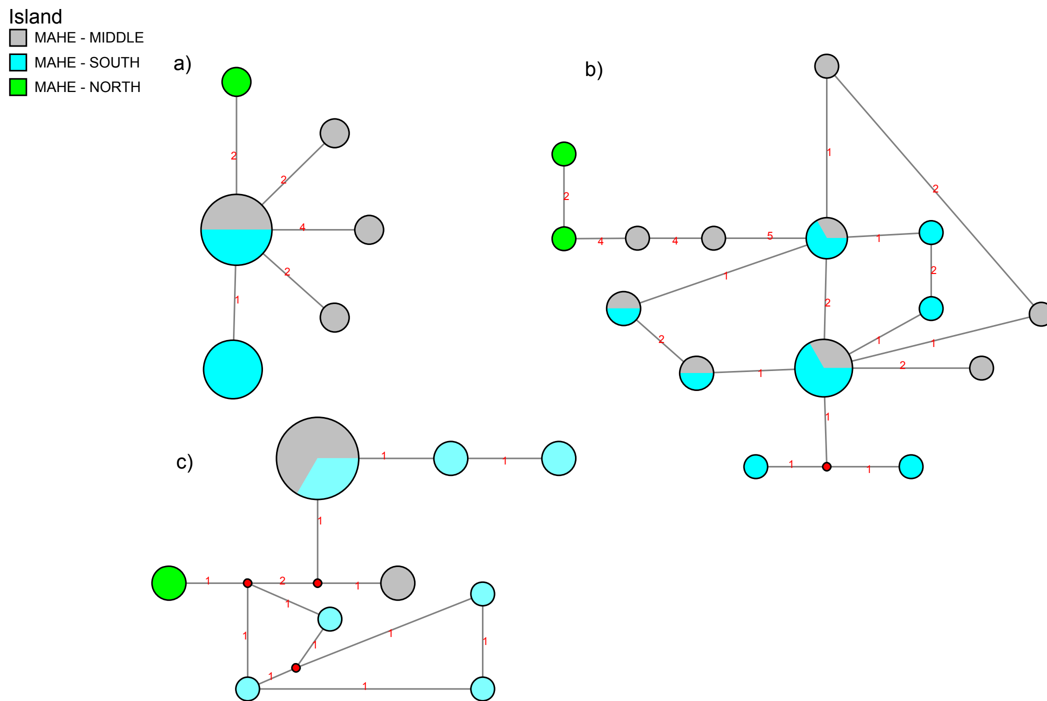

Fig 8. Median-joining haplotype networks for *Hypogeophis brevis* sensu stricto in the three nuclear loci that showed intraspecific variation: a) *brev5*, b) *pomc*, c) *rost5.* Colours in the key refer to locality (as in Fig. 3) origin of each specimen.

4.3.3.6 Intraspecific variation – Hypogeophis rostratus

No haplotypes for *H. rostratus* are restricted to single islands or larger within-island regions except for the samples from Frégate for *brev5* (Fig. 9). For *brev5* there is a widespread northern island haplotype and widespread southern island haplotype. Specimens from the eastern island of Frégate and one specimen from the northern island of Curieuse are more similar to southern island individuals. For *pomc* there is a main northern- and southern-island group cluster, however, samples from the southern island Cerf share alleles with two widespread northern island haplotypes; a pattern also observed in the widespread northern island haplotype in *rost5*. In *bdnf* and *rost5* allele sharing is extensive and there are no clear separations between populations from northern- and southern- island groups. For *pomc* specimens

from the islands of Mahé, Silhouette and Ste Anne all have unique haplotypes and none of which are shared with other island populations.

The standardised *p*-distance NeighborNet analysis for the full nuclear dataset recovers three main clusters (Fig. 12). These groups comprise northern (Praslin, La Digue, Félicité and one specimen from Curieuse RAN31680, southern (Mahé, Silhouette and Ste Anne) and mixed (Frégate, Cerf and RAN31679 from Curieuse) groups. In the PCA plot (Fig. 11) there are two clusters, with specimens from the mixed group in the NeighborNet analysis nested within the southern group.

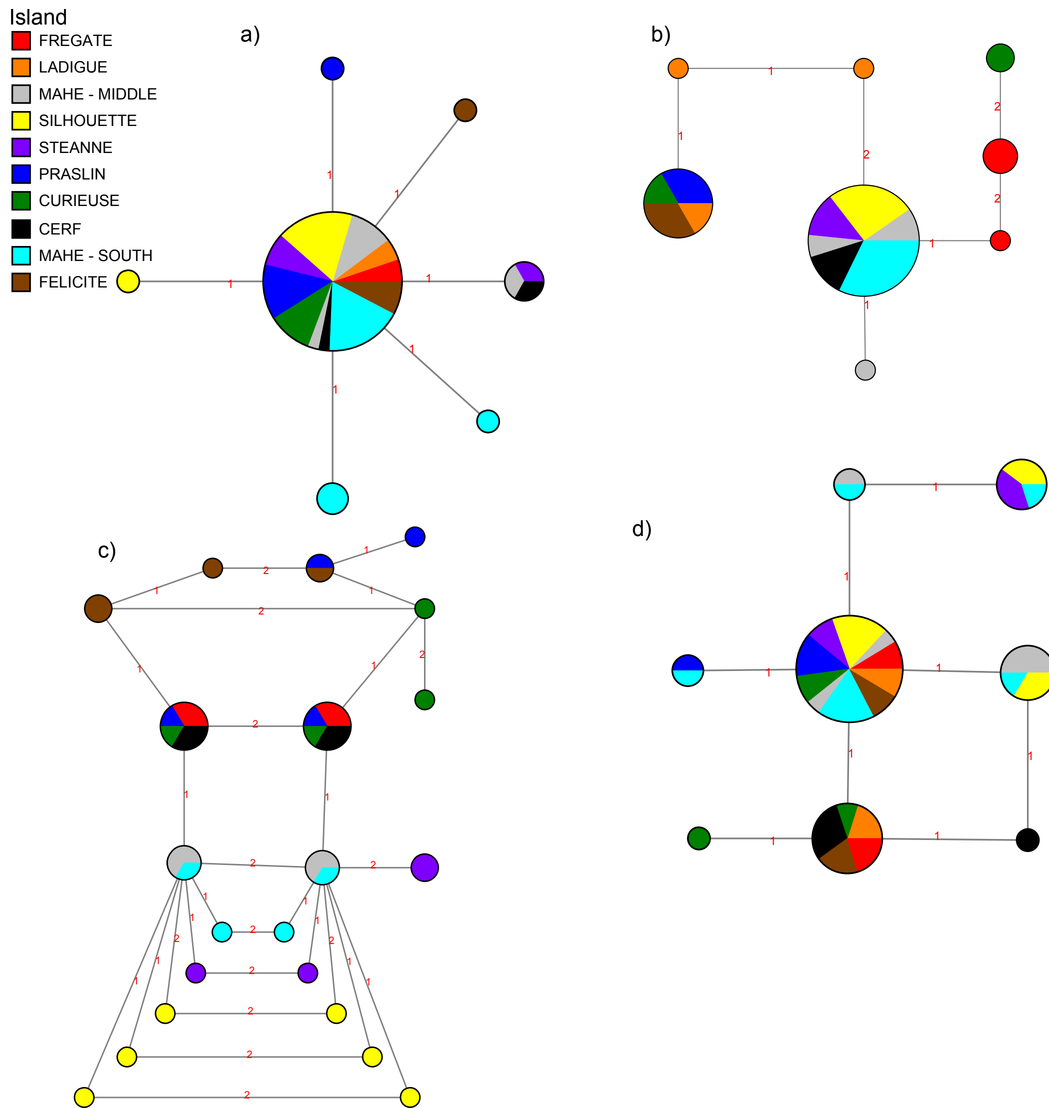

Fig 9. Median-joining haplotype networks for *Hypogeophis rostratus* in four nuclear loci: a) *bdnf*, b) *brev5*, c) *pomc*, d) *rost5.* Colours in the key refer to locality (as in Fig. 3) origin of each specimen.

4.3.3.7 Intraspecific variation – Praslinia cooperi

Some specimens of *P. cooperi* from Silhouette have unique haplotypes for *brev5*, *pomc* and *rost5* (Fig. 10). However, there is no clear overall geographic structure in the nuclear loci variation within the species. Specimen RAN31309 from Mahé has a 3bp indel in *brev5* not seen in other specimens.

No clear geographic structuring was found in the standardised *p*-distance analyses for the full nuclear dataset (Figs. 11, 12).

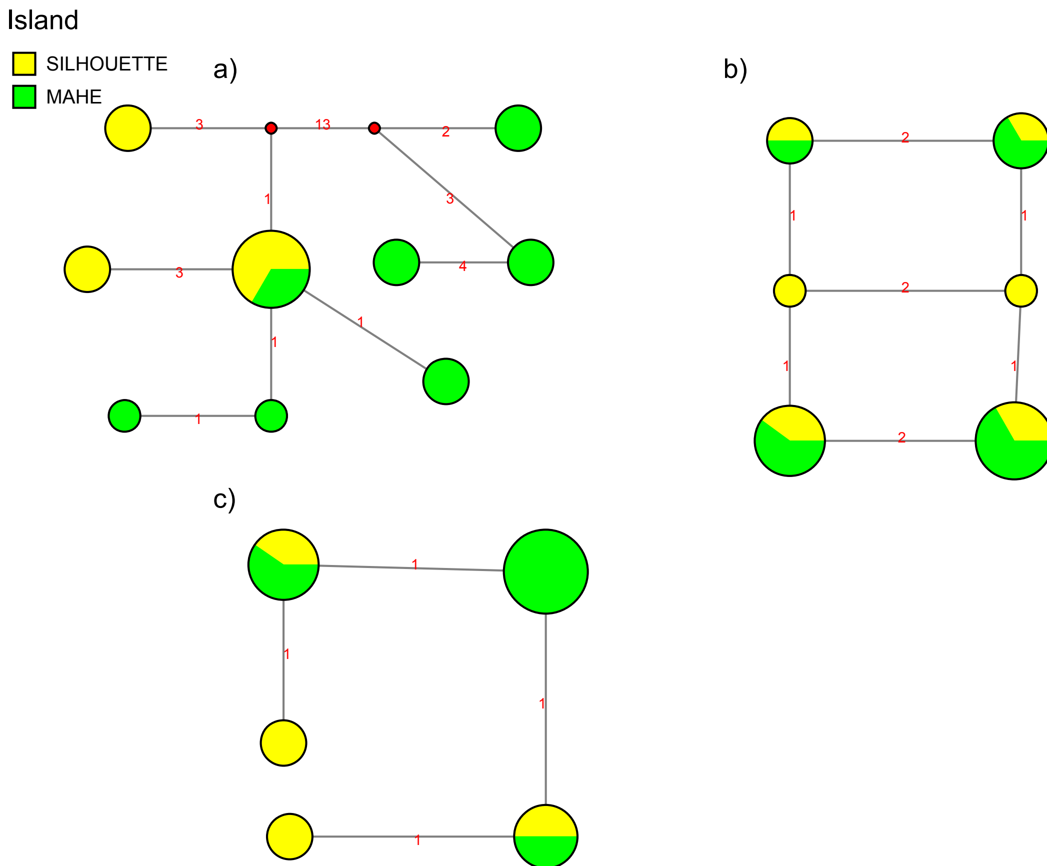

Fig 10. Median-joining haplotype networks for *Praslinia cooperi* in the three nuclear loci that showed intraspecific variation: a) *brev5*, b) *pomc*, c) *rost5* Colours in the key refer to locality (as in Fig. 3) origin of each specimen.

4.3.4 Isolation by distance

Except for *Praslinia cooperi,* the isolation by distance Mantel tests reject the null hypothesis that isolation by distance is not the cause of the intraspecific geographic patterns in the observed genetic diversity across Seychelles caecilians (Table 4). *Grandisonia alternans, G. larvata* and *H. rostratus* show strong evidence for isolation by distance, with maximal *p*-value scores. *Grandisonia alternans* has the highest observed *r* score, a reflection of

the relatively strong observed geographic structuring that was observed in other analyses.

Hypogeophis brevis only just meets the threshold of a significant *p*-value (0.049). The greatest genetic distance within this species lies between the only specimen sampled from northern Mahé (SM193) and the remaining populations, despite this northernmost locality being only ~3.5km from the population on the southern slopes of the Morne Seychellois National Park. Specimens from the latter locality are, however, genetically more similar to specimens from La Reserve ~8km further south. This geographic-genetic discordance is a likely reason for the weakly rejected null hypothesis that *H. brevis* shows no isolation by distance.

Table 4 Mantel test results between standardised genetic and geographic distances for each Seychelles caecilian species distributed across multiple sampling localities. *Z* = Mantel test Z statistic, *r* = correlation coefficient, *P*-value = significance value.

Species	*Z*	*r*	*P*-value
G. alternans	12,581,350.4207	0.5659	0.0001
G. larvata	4,203,326.7104	0.2233	0.0001
G. sechellensis	3,792,041.9140	0.3651	0.0008
H. brevis sensu stricto	201,643.4186	0.2301	0.0049
H. rostratus	4,550,721.6922	0.3399	0.0001
P. cooperi	333,935.4582	0.0938	0.1928

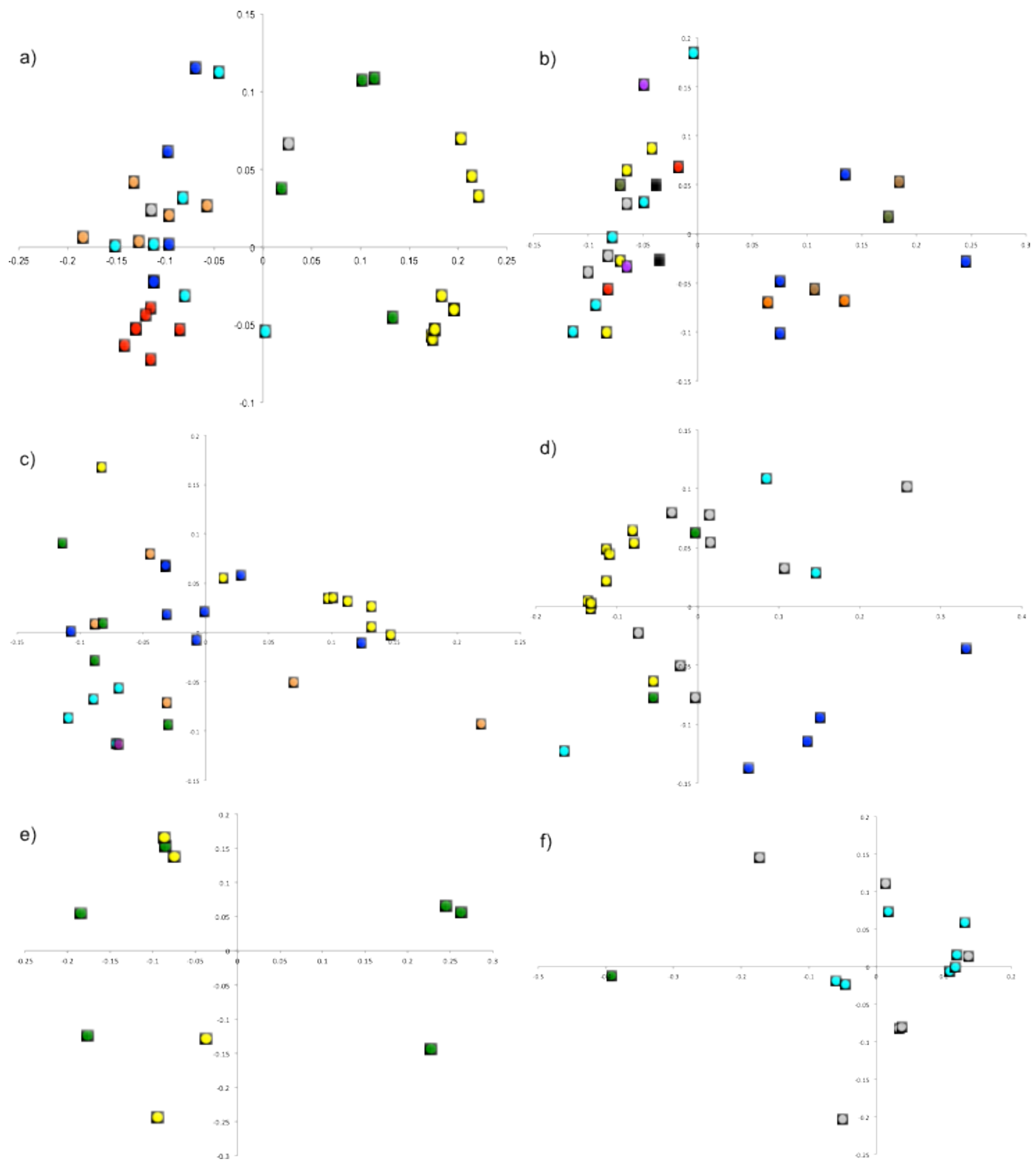

Fig 11. PCA plots based on multilocus standardised *p*-distances for each of the Seychelles caecilians: a) *Grandisonia alternans*, b) *Hypogeophis rostratus*, c) *G. larvata*, d) *G. sechellensis*, e) *Praslinia cooperi*, f). *H. brevis*. Colours correspond to localities used in Fig. 3.

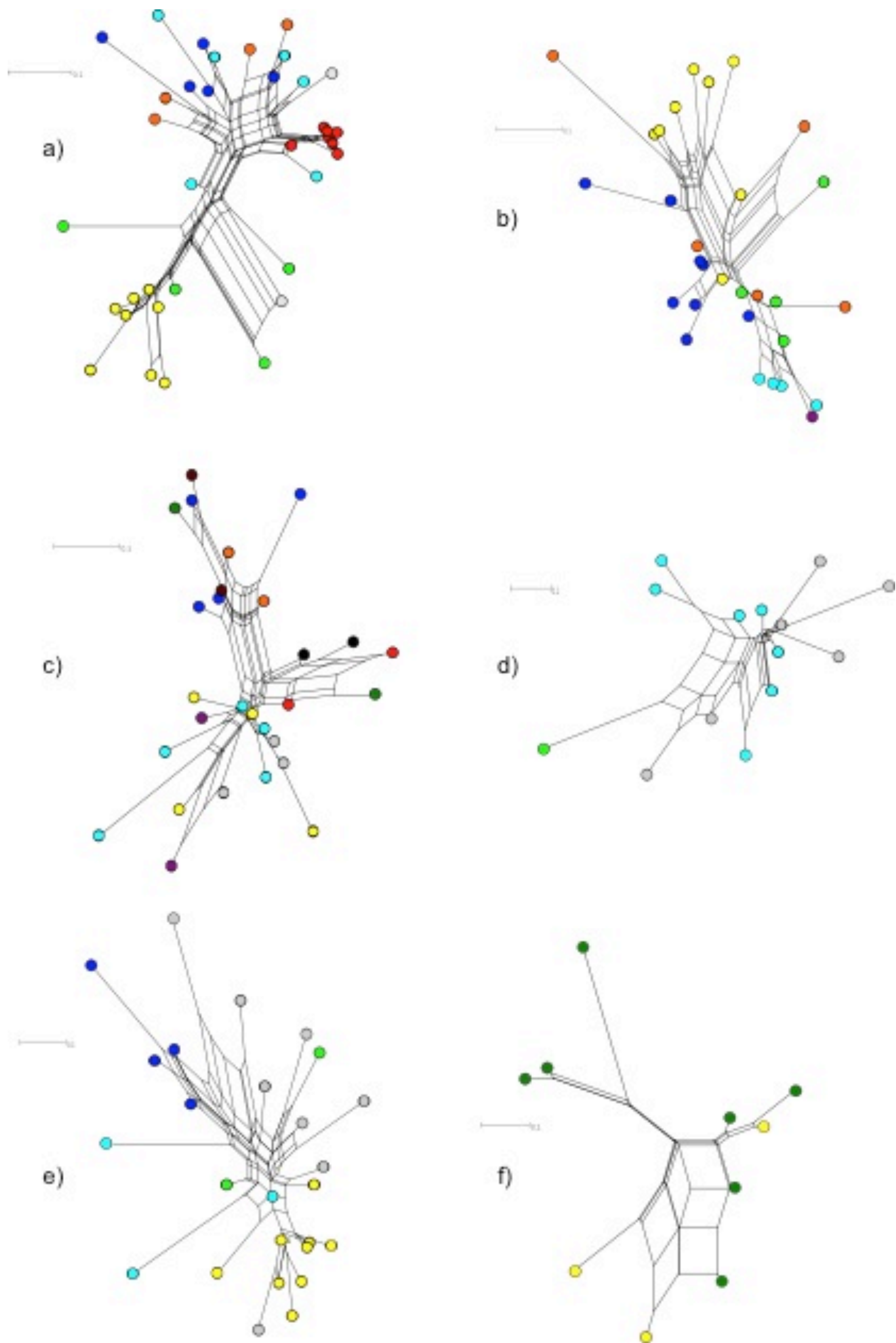

Fig 12. Splitstree NeigborNet inferred from multilocus standardised *p*-distances for each of the Seychelles caecilians: a) *Grandisonia alternans*, b) *G. larvata*, c) *H. rostratus*, d) *H. brevis*, e) *G. sechellensis*, f) *Praslinia cooperi*. Colours on tips of branches correspond to localities used in Fig. 3.

Table 5 Patterns of geographic structuring observed between the Seychelles caecilians. If shading of boxes is blue this indicates that the pattern was observed in both molecular and morphology (see Chapter 3); red indicates only molecular; and grey indicates only morphology. The North and South headings refer to the northern and southern-island groups. These are used broadly and if there is substructure within these then they are indicated in other cells. See Fig. 13 for a graphical representation.

	North	**South**	**North + south**	**Mahé**	**Silhouette**	**Frégate**	**Praslin**	**Mahé + Frégate**	**N. Mahé**	**No pattern**
G. alternans	[grey]	[grey]	[red]		[blue]	[red]		[grey]	[red]	
G. larvata	[grey]	[grey]	[red]	[grey]	[blue]					
G. sechellensis	[red]	[red]					[red]			[grey]
H. brevis group	[blue]	[blue]		[blue]			[blue]			
H. rostratus	[blue]	[blue]				[blue]				
P. cooperi										[blue]

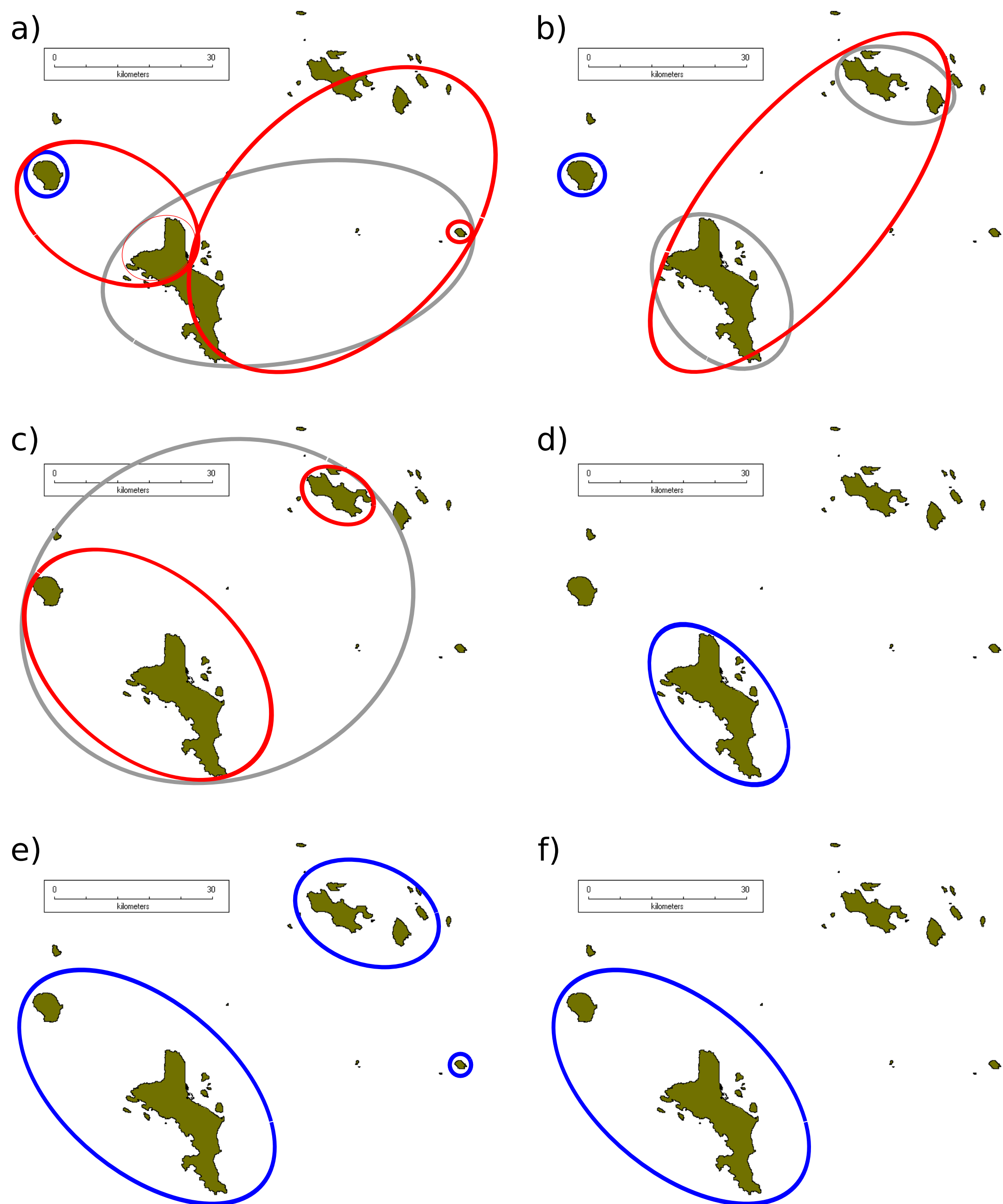

Fig. 13 Maps of the granitic Seychelles showing the patterns of clustering in each species of caecilian studied in this chapter: a) *Grandisonia alternans*, b) *G. larvata*, c) *G. sechellensis*, d) *Hypogeophis brevis*, e) *H. rostratus*, f) *Praslinia cooperi*. Blue indicates that the pattern was observed in both molecular and morphology (see Chapter 3); red indicates only molecular; and grey indicates only morphology.

4.4 Discussion

This study provides the most extensive phylogeographic insight into closely related, co-distributed taxa in the Seychelles and the largest comparative population-level study of caecilians globally. It adds to knowledge of how amphibians, especially caecilians, evolve on islands and to the documentation and understanding of patterns of intraspecific diversity across the granitic Seychelles. Varying levels of genetic variation exist among the different caecilian species, and those with the deepest intraspecfic diversity (*G. alternans, G. larvata* and *Hypogeophis rostratus*) are the most widespread and show the highest levels of geographic structuring, a pattern also observed in morphology (Chapter 3).

Contradictory patterns do however appear in the molecular data among the different species that occur on more than one island (summarised in Table 5). This incongruence is highlighted in that two multi-island species show no geographic variation (*G. sechellensis* based on mtDNA, and *P. cooperi*), *H. rostratus* shows a basal northern- vs southern-island split, *G. alternans* and *G. larvata* reveal a geographic split between specimens from Silhouette and elsewhere, and independence of each island population in *G. sechellensis* based on nuDNA. This variation in patterns contrasts strongly with the generally uniform pattern found in Seychelles lizards (Rocha *et al.* 2010b; a, 2011, 2013a; Valente *et al.* 2014). Thus, hypotheses A, B, C and D outlined at the end of the Introduction are rejected as universally true of Seychelles caecilians. Additionally, the universality of hypothesis E can also be rejected because the ecological specialist *P. cooperi* (restricted to higher altitudes of the highest islands and possessing the most well developed aquatic larval

stage) lacks geographic structuring of its genetic variation whereas the greatest ecological generalist and the most widely distributed species (*H. rostratus*) displays substantial geographic genetic structure. It is particularly notable that different geographic structuring occurs among co-distributed taxa that have presumably broadly encountered the same environmental histories.

In three of the Seychelles caecilians (*H. brevis* group*, H.rostratus* and *P. cooperi*) similar patterns of geographic variation are observed between molecules and morphology. However, contrasting patterns are observed in *G. alternans, G. larvata* and *G. sechellensis*. It is unclear exactly what has caused the contrasting patterns of intraspecific variation among the Seychelles caecilians, though it is unlikely to be readily explained by any single factor alone.

The mitochondrial-nuclear discordance found in *G. sechellensis* (endemic to Mahé, Silhouette and Praslin) is a notable finding. The mitochondrial data indicate that there is no clear geographic structuring among populations of *G. sechellensis* (similar to patterns found in morphology, Chapter 3) on these three islands, similar to the pattern found in the Seychelles treefrog (Chapter 2; Maddock et al., 2014). The nuclear loci support some degree of independence of the populations from each island, similar to the pattern found for sooglossid frogs (Taylor *et al.* 2012) and freshwater crabs (Daniels 2011). This mitochondrial-nuclear discordance is possibly explained by mitochondrial introgression, perhaps as recently as during the last glacial maximum ~10Ka, when all of the islands would last have been connected (Camoin et al. 2004; Colonna et al. 1996; Rohling et al. 1998; Siddall et al. 2003). Due to the matrilineal method of inheritance and lower

effective population size of mtDNA compared to nuDNA it is possible for mitochondrial introgression to occur in recently connected populations that have been in contact (e.g. Bastos-Silveira et al., 2012; Melo-Ferreira et al., 2012). Mitochondrial introgression has previously been found in skinks of the genus *Mabuya* in the Seychelles (S. Rocha unpublished data) although this was between two (sister) species on the southern island of Mahé, with the same two species in the northern islands remaining genetically distinct.

Grandisonia alternans and *G. larvata* have similar distributions and patterns of geographic structuring of genetic variation. Both of these species (not sister taxa: see Chapters 5 and 6) have a deep genetic split between specimens from Silhouette and from the remaining islands that is not observed in other Seychelles caecilians. A common abiotic event explaining this divergence can be ruled out because they are demonstrably non-contemporaneous. However, the deep divergences might nonetheless have a common cause in terms of a break in gene flow enforced by isolation because of the relatively deep ocean trench separating Silhouette (and the small island of North) from the remaining islands (Rocha et al., 2013). A similar pattern has been observed in the freshwater crab *Seychellum alluaudi* (Daniels 2011) and the sooglossid frog *Sooglossus sechellensis* (Taylor *et al.* 2012) suggesting that this deep trench likely influences patterns of structuring across species that may be more influenced by saltwater barriers. Contrastingly, in both species, morphology supports a Silhouette, a northern- and a southern island split (Chapter 3), suggesting that ecological adaptation has overprinted evolutionary history in these two cases.

In *G. alternans*, unlike *G. larvata,* there is notable intra-island structuring of genetic variation, with specimens in the northern region of Mahé being most closely related to specimens from Silhouette. Although *G. alternans* has been found at heights of >700 m/asl, dispersal might have been reduced by the Morne Seychellois National Park (MSNP) mountain range (occurring across the boundary of northern and mid-Mahé). The MSNP is the highest mountain range in the Seychelles with peaks as high as 905 m/asl. Within the highest reaches of MSNP habitat is varied and is unlike other habitat seen throughout the Seychelles which coupled with a reduction in species and population sizes at altitude (e.g. Tolhurst et al., *in press*) may limit dispersal capabilities. The genetic data indicate that *G. alternans* from the northern- and mid-Mahé groups could be interbreeding in the southern portion of the MSNP, but more detailed population genetics research will be needed to test this.

Hypogeophis brevis, although not occurring on Silhouette, shows a similar pattern of genetic variation on Mahé to that observed in *G. alternans,* with the only sample collected from northern Morne Seychellois National Park being genetically distinct from the other populations occurring further south. To my knowledge, partial breaks in gene flow across the northern part of Mahé found in *H. brevis* and *G. alternans* have not been reported in any other Seychelles organisms. More detailed examinations of genetic variation in other organisms are needed to test the generality of this pattern across multiple taxonomic groups.

The lack of geographic structure in genetic variation in *Praslinia cooperi* was unexpected because this appears to be one of the more ecologically specialised Seychelles caecilian species. A causal explanation is perhaps

found in its unusual (for the Seychelles) life-history mode. *Praslinia cooperi* has an extended, fully aquatic larval stage and adults are generally found close to flowing water bodies. It is possible that during very heavy rains this species is washed out over large distances, potentially even between islands in freshwater films on top of the sea (e.g. Measey et al., 2006). Circumstantial evidence in support of this speculative hypothesis is found in the Seychelles tree frog – this is the only other native Seychelles amphibian species with a fully aquatic larval stage and, interestingly, it also lacks clear island-based structuring of its genetic variation (Chapter 2; Maddock et al., 2014).

The two species that show the largest within-island amounts of geographic structuring, *G. alternans* and *H. brevis,* are the two species for which reproductive strategy is unknown (San Mauro *et al.* 2014). During recent fieldwork, nests with eggs of both *G. alternans and H. brevis* were discovered (pers. obs.) but it was not possible to determine whether the species hatch as fully developed young or if they have a larval stage.

The deep split (but sister relationship between) *H. brevis* sensu stricto and *H*. cf. *brevis* CR may be caused by habitat differences at least as much as by altitude (730 m/asl in *H*. cf. *brevis* CR vs. 520 m/asl in *H. brevis*). Congo Rouge, along with the highest peaks of MSNP, contains unique habitats for the Seychelles, with the vegetation dominated by very wet forests with lots of epithetic growth and endemic vegetation (Procter 1984).

The three dwarf species (*H. brevis* group) are the only Seychelles caecilians restricted to single islands. The extremely pointed snouts and dwarfism of the *H. brevis* group (see Fig. 1.5 for example) suggests that they are ecological specialists, however, data on their ecologies and life histories is

poorly known. If we assume that the dwarf species are ecological specialists then it is likely that dispersal ability will be greatly reduced. This reduced dispersal ability hypothesis can be supported by their limited distributions, and by the geographic proximity of *H. brevis* sensu stricto and *H.* cf. *brevis* CR which suggests that gene flow can even break down between geographically close populations.

Why, unlike other organisms, do co-distributed Seychelles caecilians have different geographic patterns of intraspecific variation? Perhaps this is a feature of ancient endemic islands radiations, especially amphibians (rare on other islands). Maybe the ecological diversity of Seychelles caeciians is much greater than currently appreciated. Beyond more extensive and intensive studies of their ecology, further insights would probably be gained by carrying out detailed population genetics studies using tools such as microsatellites (e.g. Barratt et al., 2011) or RAD-seq (e.g. Davey et al., 2010). For example, insights into fluctuations in population size and into levels of gene flow would greatly enrich available data sources and help to discriminate among explanatory hypotheses.

Based on molecular and morphological (Chapter 3) patterns of variation within Seychelles caecilians the data supports the recognition of the six nominal species and the two undescribed dwarf species, *H.* cf. *brevis* CR and *H.* cf. *brevis* Praslin. Though suggestions that genetic splits of less than those observed in *G. alternans, G. larvata* and *H. rostratus* be recognised as candidate species in amphibians (e.g. Fouquet *et al.* 2007; Funk *et al.* 2012), they show several conflicting patterns in molecular and morphological data and they should therefore be considered to be unconfirmed candidate species.

Even in the species with the largest genetic split, *G. alternans* (7.6% sequence divergence), there is possible hybridisation of specimens from different mitochondrial lineages on the southern slopes of MSNP, Mahé. This possible hybridisation suggests that recognition of these lineages as distinct species units may be premature, especially since morphology does not fully support molecular data. However, hybridisation in sympatric species has been reported as common in other radiations (e.g. Lamichhaney *et al.* 2015; Ford *et al.* 2015) and therefore it is possible that the genetic split in *G. alternans* is a distinct species. Further work is needed in order to fully establish whether these lineages require recognition as independent species but until then the unique geographically structured lineages in each species should be considered to be an evolutionary significant unit.

CHAPTER 5

NEXT-GENERATION MITOGENOMICS: A COMPARISON OF APPROACHES APPLIED TO CAECILIAN AMPHIBIAN PHYLOGENY

Abstract

Mitochondrial genome (mitogenome) sequences are being generated with increasing speed due to the advances of next-generation sequencing (NGS) technology and associated analytical tools, yet detailed comparisons of alternative approaches applied to the same taxa have not been undertaken. To explore the utility of alternative approaches we compared a 'traditional' Sanger sequencing method with two NGS approaches (shotgun sequencing and non-indexed, multiplex amplicon sequencing) on four different sequencing platforms (Illumina's HiSeq and MiSeq, Roche's 454 GS FLX, and Life Technologies' IonTorrent) to produce seven (near)-complete mitogenomes from six species that form a small radiation of caecilian amphibians from the Seychelles. The fastest, most accurate method of obtaining mitogenome sequences that we tested was direct sequencing of genomic DNA (shotgun sequencing) using the MiSeq platform. Bayesian inference and maximum likelihood approaches for seven different partitioning strategies were unable to resolve all phylogenetic relationships robustly among the Seychelles caecilian species, indicating the need for additional data in this case.

5.1 Introduction

Technological advancement and decreasing costs have increased the use of high-throughput sequencing platforms in evolutionary biology (see van Dijk *et al*. 2014). Recently, several studies have generated mitogenomic data sets for phylogenetics using next-generation sequencing (NGS) (e.g., Gillett *et al.* 2014; Groenenberg *et al*. 2012; Lloyd *et al*. 2012; Timmermans *et al*. 2010), though variably with long-range PCRs (e.g. Lloyd *et al*. 2012) or shotgun sequencing (e.g. Gillett *et al*. 2014) and using a variety of sequencing platforms. Detailed comparisons and evaluations of different NGS approaches for mitogenomic phylogenetics of the same set of taxa have not been carried out.

Here we present a comparison of four different NGS approaches for generating (near-)complete mitogenome DNA sequences. Two primary methods were employed: (1) multiplex sequencing of pooled, non-indexed long-range PCR products from a multitude of taxa (e.g. see Timmermans *et al*. 2010) using three different platforms: HiSeq (Illumina), 454 GS FLX (Roche), and IonTorrent (Life Technologies), and (2) individually indexed shotgun sequencing of genomic DNA (e.g. see Gan, Schultz, & Austin, 2014) using the MiSeq platform (Illumina).

We explored the efficacy of various approaches for generating complete mitogenome DNA sequences for a clade of caecilian amphibians (Gymnophiona) endemic to the Seychelles. Mitogenomic data have played an especially important role in recent advances in the understanding of caecilian phylogeny, systematics, and evolution (San Mauro *et al*. 2009; San Mauro *et al*. 2012, 2014; San Mauro, Gower, Oommen, Wilkinson, & Zardoya, 2004;

Zardoya & Meyer, 2000; Zhang & Wake, 2009). Caecilian mitogenomes have also provided the best evidence for tandem duplication and random loss as a mechanism of mitochondrial gene order rearrangements (San Mauro *et al.* 2006). However, mitogenomes have only partly been applied, thus far, to the ongoing problem of the relationships among the Seychelles caecilians. The Seychelles caecilians comprise a radiation (Gower *et al.* 2011; Hedges, Nussbaum, & Maxson, 1993; Nussbaum, 1984; Kamei *et al.* 2012; Roelants *et al.* 2007; Wilkinson *et al*. 2002) of six nominal species in three genera (*Grandisonia alternans, G. larvata, G. sechellensis, Hypogeophis brevis, H. rostratus, Praslinia cooperi*) within the family Indotyphlidae (following the classification of Wilkinson *et al*. 2011). Prior to 2009, analyses of small fragments of mtDNA sequence data had reached no consensus beyond that the monotypic *Praslinia* is sister to all other Seychelles species (Gower *et al*. 2011; Hedges *et al*. 1993; Loader *et al*. 2007; Wilkinson *et al.* 2002, 2003). More recently, complete (San Mauro *et al*. 2014) or near-complete (Zhang & Wake, 2009) mitogenomes have been generated for four of the Seychelles species, but this limited taxon sampling precluded comprehensive phylogenetic insights. Resolution of the phylogenetic relationships among the Seychelles caecilians would be beneficial in helping to stabilise their genus-level taxonomy (see Wilkinson *et al.* 2011), and in providing a platform for more detailed analysis of the evolution of reproductive traits within indotyphlids, which likely includes the re-evolution of a larval stage (San Mauro *et al.* 2014).

5.2 Methods

5.2.1 Taxon sampling and DNA extraction

Six Sanger-sequenced complete or near-complete mitogenome sequences had been previously generated for four of the six nominal species of Seychelles caecilians (San Mauro *et al.* 2014; Zhang & Wake, 2009) (see Table 1). These mitogenomes were generated using multiple primer pairs designed to amplify 14 (San Mauro *et al*. 2014) or 13 (Zhang & Wake, 2009) overlapping fragments. We attempted to generate sequences of a further eight mitogenomes for five Seychelles species using four NGS approaches. Samples were obtained from the University of Michigan Museum of Zoology, USA (voucher specimen codes with the abbreviation UMMZ) or samples deposited at UMMZ and collected by RAN (voucher specimen codes with the abbreviation RAN). For three individuals (*G. alternans* UMMZ240022, *G. larvata* UMMZ240023*, H. brevis* UMMZ192977), mitogenomic data were generated using more than one method. Our sampling (Table 1) included the two Seychelles caecilian species (*G. alternans*, *H. brevis*) not previously sampled for mitogenomes and whose sister taxa are not resolved (Gower *et al*. 2011; Loader *et al*. 2007; Wilkinson *et al*. 2002, 2003).

Liver and/or muscle samples of Seychelles caecilians were obtained during fieldwork between 1988 and 1991. Tissues were frozen at -80°C and voucher specimens fixed in formalin and stored in 70% EtOH at the University of Michigan Museum of Zoology, Ann Arbor, USA (UMMZ). Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN), following manufacturer’s guidelines with the exception of the final suspension solution,

which was modified to 2x100µl of buffer AE (the first elution was used in all subsequent analyses).

5.2.2 gDNA shotgun sequencing using the MiSeq (Illumina) platform

Next-generation sequencing libraries for six individual samples (two *G. alternans*; one of each of *G. larvata, G. sechellensis*, *H. brevis* and *H. rostratus*), destined for shotgun sequencing, were prepared for Illumina MiSeq sequencing using a standard Illumina Nextera DNA kit. The primary aim of this sequencing run was to develop anonymous nuclear markers (see Lewis *et al*. 2014). Paired-end reads (≤251bp long) were sequenced using a 500 cycle v.2 reagent kit on a single MiSeq flow cell. Each sample was indexed so that all sequences could be individually identified.

The paired-end MiSeq data were combined for each sample and subsequently cleaned with the Trim Ends function in Geneious v.6.1.4 (Biomatters) using default settings. FASTQ files containing the paired-end data were run through the MITObim pipeline (100 iterations; --quick option) using the six previously published Seychelles caecilian mitogenomes (San Mauro *et al*. 2014; Zhang & Wake, 2009) as a reference. MitoBim was chosen because of its superiority over other mapping tools (Hahn *et al*. 2013). However, initial runs for each sample yielded reconstructed mitogenomes with approximately 500 base pairs (bp) missing from the end of the assembly. To combat this, 1,000bp of the linear reference mitogenomes were moved from the end to the start of the alignment and analyses were rerun. Both runs for each specimen were then compared, aligned against each other, trimmed, and a consensus sequence was produced in Geneious.

5.2.3 Multiplex amplicon sequencing using HiSeq (Illumina), 454 GS FLX (Roche) and Ion Torrent (Life Technologies) platforms

The complete mitogenomes of *G. alternans* (UMMZ240022) and *H. brevis* (UMMZ192977) along with the partial mitogenome (6471 bp) of *G. larvata* (UMMZ240023) were sequenced in parallel with 470 non-indexed long-range mitogenomic PCR amplicons from 270 other animal taxa (including some caecilians), as part of a larger project.

Long-range PCRs were carried out in 50 µl reaction volumes using the Expand 20kb^{PLUS} PCR System (Roche) using 4 µl of gDNA following manufacturers' recommendations. The mitogenomes were amplified in two overlapping fragments, ~6.4kb and ~10.7kb, using the primer pairs Amp-12S.F (5'-AAGAAATGGGCTACATTTTCT-3') + Amp-P3.R (5'-GCTTCTCARATAATAAATATYAT-3') and Amp-P4.F (5'-GGMTTTATTCACTGATTYCC-3') + Amp-12S.R (5'-TCGATTATAGAACAGGCTCCTCT-3') (San Mauro *et al*. 2004), respectively, however, the ~10.7kb fragment failed to amplify for *G. larvata* (UMMZ240023). Because of the degeneracy of primers Amp-P3.R and Amp-P4.F, 4 µl of 10 µM primer were added to each reaction, whereas only 2 µl were used for Amp-12S.F and Amp-12S.R. The PCR cycling profile for Amp-12S.F + Amp-P3.R was as follows: initial denaturation for 2 min at 92 °C, followed by 10 cycles of 15 s at 92 °C, 30 s at 45 °C, 4 min at 68 °C, followed by 30 further cycles in which the extension time was lengthened by 10 s per cycle, and terminated with a final extension of 10 min at 68 °C. The PCR cycling profile for Amp-P4.F + Amp-12S.R was as follows: initial denaturation for 2 min at 92 °C, followed

by 10 cycles of 15 s at 92 °C, 30 s at 48 °C, 9 min at 68 °C, followed by 30 further cycles in which the extension time was lengthened by 10 s per cycle, and terminated with a final extension of 10 min at 68 °C. PCR products were purified using QIAquick PCR Purification Kit (QIAGEN) and quantified using a NanoDrop spectrophotometer (Thermo Scientific). An equimolar solution of all 475 amplicons was prepared for NGS sequencing using the Illumina HiSeq, Roche 454 and Ion Torrent platforms. Short fragments of mtDNA (*12S* and *16S* rDNA, *cox1*, *cytb*) that had been Sanger sequenced for each species (Gower *et al*. 2011; Hedges, Nussbaum, & Maxson, 1993) were used as seeds for read assembly (see below) and to provide amplicon identity.

5.2.4 Initial reduction of Illumina HiSeq dataset

Because the Illumina HiSeq platform produces a vast amount of data (and because the samples were not individually indexed), the full dataset, which consisted of 270 individual animals, was subjected to an initial reduction to facilitate mitogenome reconstruction for Seychelles caecilians. Three previously published (Sanger-sequenced) Seychelles caecilian mitogenomes (*G. sechellensis, H. rostratus, P. cooperi*; GenBank accessions KF540152, KF540152, KF540162 respectively) plus one of a proximate outgroup (the Indian indotyphlid *Indotyphlus maharashtraensis*, GenBank accession KF540157) were aligned using Muscle (Edgar 2004b) in Geneious with default settings. The alignment was checked by eye and obvious mistakes corrected manually.

The alignment was then viewed in Geneious with a sliding window in order to identify regions with similar magnitudes of sequence (dis)similarity

among the four mitogenomes. Separate sub-alignments were generated for each of 16 such regions, the sub-alignments ranging in size from 289–2,525bp (each overlapping by at least 50bp with neighbouring alignments to counter potential loss of reads) (Table 3). The maximum sequence divergence (p-distance) among the four mitogenomes was calculated from the sliding window for each of the 16 sub-alignments. A consensus sequence was generated for each sub-alignment. These sub-alignments were then used as references for mapping assemblies in order to extract caecilian reads from the raw, non-indexed HiSeq data, using a mismatch threshold of the maxmimum divergence among the four mitogenome sequences in each sub-alignment, plus an additional 10% allowance, per read. Reference assemblies were carried out in Geneious with the following parameters: single iteration mapping assembly, 15% gaps allowed per read, maximum gap size 50, word length 14, index word length 12, maximum ambiguity 4 (allowing 1 ambiguous base per read) and the number of mismatches allowed per read as described above. From this point, these initially reduced Illumina HiSeq data were subject to the same treatment as the Roche 454 and Ion Torrent data.

5.2.5 Mitogenome reconstruction from Roche 454, Ion Torrent and distilled Illumina HiSeq data

Each of the three amplicon data sets were assembled in Geneious using the "map to reference" function with the four Sanger sequenced seeds used as references (see above). The assemblies were performed for 100 iterations with the following settings: 3% mismatches per read, maximum gap size of 15,

maximum overlap identity of 80%, maximum ambiguity 1, and multiple best matches mapped randomly.

In order to locate relevant reads that might have been discounted in assemblies generated from the starting Sanger seeds (especially for the lower-coverage Ion Torrent data), we used mitogenomes of the same species (previously published Sanger-sequenced data available in every case, except MiSeq indexed for *G. alternans*) as references for the “map to reference” option in Geneious, and used the same settings described in the previous paragraph, except for a maximum mismatches per read of 1% and maximum ambiguity of 2. These setting modifications were applied in order to accommodate intraspecific variation.

5.2.6 Mitogenome annotation and alignment

Alignments of mitogenomic data generated from different platforms for single specimens (available for three specimens: *G. alternans* UMMZ240022, *G. larvata* UMMZ240023*, H. brevis* UMMZ192977) were created using the de-novo assembler in Geneious v.6.1.6. No major errors were detected by eye and a consensus sequence for each specimen was accepted as the final sequence for further annotation and analysis.

The six previously published (Sanger-sequenced) Seychelles caecilian mitogenome sequences were aligned using Muscle in Geneious with default settings; any obvious misalignments within tRNA genes were corrected manually. The newly generated sequences were then added and aligned using Geneious Consensus Align, maintaining existing gaps, with 70% similarity, gap open penalty of 12, and a gap extension penalty of 3. All novel

mitogenomes were compared with those previously published and Sanger seeds (see above) to increase the likelihood of correct reconstruction of the data. When checked, only the new tRNA gene sequences had (very small) obvious mistakes that were attributable to misalignment rather than sequencing or reconstruction error, and these were sought and removed using GBlocks (Castresana 2000) using the 'with half' setting.

The initial annotation of the newly reconstructed mitogenomes was carried out using MITOS (Bernt *et al.* 2013), BLASTn, and by alignment against the six previously published Seychelles caecilian mitogenomes (San Mauro *et al*. 2014; Zhang & Wake, 2009). The final annotation was undertaken manually in Geneious. When annotating protein-coding genes, information was incorporated from codon position determined using MEGA v.6.06 (Tamura *et al*. 2013). GenBank accession numbers for newly generated sequences can be found in Table 1.

5.2.7 Phylogenetic analysis

Following San Mauro *et al*. (2009, 2012, 2014), the regulatory, non-coding L-strand replication and control regions were removed from the alignment. Best-fit models of nucleotide substitution and data-partition schemes were determined using PartitionFinder v.1.1.1 (Lanfear *et al*. 2012) for five datasets, comprising all or subsets of the concatenated first, second and third codon positions of protein coding genes, concatenated rRNA genes, and concatenated tRNA genes (total of 15,399 aligned bp excluding ambiguously aligned sites which were removed) (see Table 5). Potential saturation of third-codon positions was assessed using the method described by Xia et al. (2003)

in DAMBE v.5 (Xia, 2013); PAUPX v.4.0a136 (Swofford 2002) was used to test for base composition heterogeneity and, where found, bootstrap (1000 replicates) LogDet/paralinear (Lake 1994; Lockhart *et al.* 1994) distance analyses using the minimum evolution algorithm with default parameters were also carried out.

Phylogenetic trees were inferred using Bayesian inference (BI) and maximum likelihood (ML) algorithms implemented in the programs MrBayes v.3.2.2 (Ronquist *et al.* 2012) and RaxML v.8.0.24 (Stamatakis, 2014), respectively and run through the Cipres Science Gateway server (Miller *et al.* 2010). For BI, the five datasets described in the previous paragraph were each subjected to two independent analyses. Optimal partitioning strategies and best-fit models as determined by PartitionFinder are given in Table 5. The BI analysis was run for 10^7generations and sampled every 10,000 generations with one cold and three heated chains. Chains were checked for convergence using Tracer v1.5 (Drummond & Rambaut 2007) by assessing ESS scores and by visualisation of mixing on the trace; the first 10% of trees were discarded as burn-in. For the ML analyses the Blackbox option was employed using default options (Stamatakis 2006).

BI of the amino acid dataset was conducted using PhyloBayes (Lartillot *et al.* 2009). PhyloBayes implements the CAT model (Lartillot & Philippe 2004) which allows for site-specific rates of mutation and is often considered a more realistic model of amino acid evolution, and being well suited to larger multigene alignments. Two independent runs were carried out implementing the CAT and the GTRCAT models. MCMC chains ran for at least 40,000 generations and convergence was assessed when the “maxdiff” parameter

was < 0.1. Approximately 25% of trees were discarded as burn-in and remaining trees were sampled every 100 generations.

Phylogenies were rooted with *Praslinia cooperi* based on prior evidence that this taxon is sister to all other Seychelles caecilians. This phylogenetic relationship has been recovered by all published analyses of molecular data (e.g. Gower *et al*. 2011; Hedges *et al.* 1993; Loader *et al.* 2007; San Mauro *et al.* 2012, 2014; Wilkinson *et al*. 2002, 2003; Zhang & Wake, 2009), except those of Pyron & Wiens (2011) and Pyron (2014), who recovered *Grandisonia alternans* as the sister group instead. We consider the latter problematic (MW, unpublished) and disregard them here.

To investigate taxon instability and any impact this might have upon support, we interrogated sets of bootstrap or Bayesian trees with the intersection algorithm described by Wilkinson (1996) and implemented in REDCON 3.0 (http://www.nhm.ac.uk/research-curation/research/projects/software/), which returns a comprehensive summary of the support (frequency of occurrence) for all full and partial (i.e., not including all taxa) splits in a set of trees. These analyses were performed on subsamples of 1,000 trees drawn randomly from the full samples of Bayesian trees.

5.3 Results

5.3.1 Next-generation mitochondrial genome sequences

Seven near-complete mitogenomes were reconstructed with varying degrees of quality and coverage. All of the trialled methods used in this study provided reasonable coverage of the mitogenomes, apart from the Ion Torrent multiplex

approach. The Illumina HiSeq multiplex data produced the greatest coverage, followed by the shotgun-sequenced Illumina MiSeq and Roche 454 data (Table 2).

For the *G. larvata* sample (UMMZ 240023), sequenced using the multiplex method, approximately only one third (i.e. 5,787 bp; see Table 1) of the mitogenome was obtained, which represented a single long amplicon. This single amplicon did however have a high coverage of reads for it – the highest of any sample when compared to the length of the final sequence (Table 2).

Of the three multiplex sequencing methods, the Ion Torrent approach was least successful. Considerably fewer reads were obtained and single phantom nucleotides were present (as determined by comparison with data generated using Illumina HiSeq and MiSeq, Roche 454, and Sanger sequencing). The phantom single nucleotides comprised between 0.28 and 0.43% of the total reconstructed sequences (Table 4). Conversely, the mitogenome of *H. brevis* (UMMZ192977), reconstructed from Roche 454 multiplex data, contained eight phantom single nucleotide insertions (as judged by comparison with data generated from the Illumina HiSeq and MiSeq, and Ion Torrent platforms used for the same sample), accounting for only 0.05% of the reconstructed sequence (Table 4). All other mitogenome reconstructions that we generated with the multiplex approach (regardless of sequencing platform) and the MiSeq shotgun sequencing approach lacked evidence of phantom nucleotide insertions. The newly generated mitochondrial genome sequences (*H. brevis* and *G. alternans*) conform to the vertebrate consensus organization (Boore, 1999; Lupi *et al*. 2010) in terms of gene content and order.

Table 1. Voucher specimen (codes refer to voucher's: RAN = RAN's field numbers; UMMZ = University of Michigan Museum of Zoology; MVZ = Museum of Vertebrate Zoology, Berkley) and associated mitogenome sequence information for the six nominal species of Seychelles caecilian (species of *Grandisonia, Hypogeophis, Praslinia*). GenBank codes in bold were published previously. bp = base pairs. If numbers of bp are only reported in the column "total" then these were generated by shotgun sequencing on the MiSeq platform. X = genome sequence not fully complete; (1) = voucher incorrectly identified as *G. alternans* by Zhang & Wake (2009: see San Mauro et al. 2014). Specimen highlighted in grey was excluded from phylogenetic analysis due to the mitogenome sequence being substantially incomplete.

Species	Voucher	GenBank code	Published	BP - total	BP - HiSeq	BP - 454	BP - IonTorrent	GC %
G. alternans	RAN31062	*Pending*	This study	16,065	-	-	-	38.5
G. larvata	UMMZ240023	*Pending*	This study		6,471	5,846	5,406	
G. larvata	RAN31203	*Pending*	This study	15,388	-	-	-	33.6
H. rostratus	RAN31219	*Pending*	This study	10,782	-	-	-	26.3
G. alternans	UMMZ240022	*Pending*	This study	14,827	14,343	14,019	10,743	36.1
G. sechellensis	UMMZ193076	*Pending*	This study	16,071	-	-	-	36.2
G. alternans	UMMZ192945	*Pending*	This study	14,836	-	-	-	36.6
H. brevis	UMMZ192977	*Pending*	This study	16,107	15,540	15,578	9,593	35.9
G. larvata (1)	MVZ258026	**GQ244470X**	Zhang & Wake, 2009.	15,209	-	-	-	34.8
H. rostratus	MVZ258025	**GQ244472**	Zhang & Wake, 2009.	16,151	-	-	-	35.8
P. cooperi	UMMZ192933	**GQ244475X**	Zhang & Wake, 2009.	15,218	-	-	-	38.4
G. sechellensis	UMMZ240024	**KF540152**	San Mauro et al. 2014.	16,094	-	-	-	36.3
H. rostratus	UMMZ240025	**KF540154**	San Mauro et al. 2014.	16,170	-	-	-	35.4
P. cooperi	UMMZ192934	**KF540162**	San Mauro et al. 2014.	16,192	-	-	-	38

Table 2. Coverage data for mitogenome sequences generated by different platforms, reported as number of sequence reads used and approximate number of bp used in reconstructions separated by a hyphen.

Species	Sample code	BP - total	MiSeq – mean 448bp	HiSeq – mean 95bp	454 – mean 523bp	IonTorrent – mean 98bp
G. alternans	RAN31062	16,065	6,008 – 2,691,584bp	-	-	-
G. larvata	UMMZ240023			442,600 – 42,047,000bp	2,481 – 1,297,563bp	264 – 25,872bp
G. larvata	RAN31203	15,388	562 – 251,776bp	-	-	-
H. rostratus	RAN31219	10,782	284 – 127,232bp	-	-	-
G. alternans	UMMZ240022	14,827		512,609 – 48,697,855bp	1,178 – 616,064bp	367 – 35,966bp
G. sechellensis	UMMZ193076	16,071	1,655 – 741,440bp	-	-	-
G. alternans	UMMZ192945	14,836	583 – 261,184bp	-	-	-
H. brevis	UMMZ192977	16,107	3,092 – 1,385,216bp	670,560 – 63,703,200bp	2,148 – 1,123,404bp	375 – 36,750bp

Table 3. Comparison of performance of five approaches for generating our mitogenome sequence data from eight samples of Seychelles caecilian. Approximate relative 'values' depicted are X = low, XX = moderate, XXX = high.

Method	Sequencing	Sample preparation time	Sample preparation cost	Sequencing running time	Sequencing cost	Mitogenome reconstruction time	Total time expenditure
Traditional	Sanger	XXX	XX	X	X	XXX	XXX
Shotgun	Illumina MiSeq	X	X	XX	XX	X	X
Multiplex	Illumina HiSeq	XXX	XX	XXX	XXX	XXX	XX
Multiplex	Roche 454	XXX	XX	XX	XX	XX	XX
Multiplex	Ion Torrent	XXX	XX	X	X	XX	XX

Table 4. Size ranges used to partition the Illumina HiSeq dataset into a manageable size based on a sliding window analysis. Position 0 refers to the start of the *trnF(gaa)* tRNA gene.

Position in alignment (bp)	Maximum sequence divergence (%)
0-1,076	22
976-2,855	21
2,779-4,036	20
3,823-5,073	24
4,973-5,461	20
5,361-7,146	18
7,043-7,837	19
7,787-8,076	27
8,004-8,753	24
8,653-9,604	19
9,537-10,328	26
10,228-11,789	24
11,689-14,214	24
14,114-15,427	21
15,327-16,440	35
16,087-354	30

Table 5. Number of single base pairs (bp) that were incorrectly called in the three long-amplicon multiplexed mitogenome sequences, as inferred from consensus reads across the sequencing platform data.

	UMMZ240023 **IonTorrent**	UMMZ240022 **IonTorrent**	UMMZ192977 **IonTorrent**	UMMZ192977 **454**
A	4	11	6	1
C	1	5	5	3
G		2	2	
T	2	12	10	2
N	8	16	6	2
Insertions added		5		
Total bp	5406	10746	9593	15540

Table 6. Summary information for mitogenome data partitions and their best-fit models. All data are for nucleotides, except "Amino Acid". CS = number of constant sites, PI = number of parsimony informative sites (PI) CP1, 2, 3 = protein-coding codon position 1, 2 and 3.

Data	Sites	CS	PI	Partitions and models
All	15,399	10,534	4,241	CP1, rRNA, tRNA (GTR+G); CP2, CP3 (GTR+I+G)
Protein Coding genes	11,272	7,224	3,425	CP1, CP2, CP3 (GTR+I+G)
tRNAs	1,600	1,241	300	GTR+I+G
rRNAs	2,527	1,927	516	GTR+I+G
Amino Acid	3,746	2,996	617	

5.3.2 Mitogenomic phylogeny of Seychelles caecilians

We found no evidence of sequence saturation, but both the protein-coding and the full nucleotide datasets showed significant base compositional heterogeneities (not shown) and were thus analysed also with LogDet distances. For each dataset and partitioning strategy, the BI and ML analyses recovered the same set of phylogenetic relationships (Fig. 1). All analyses agreed in providing maximal support for each species that was represented by more than one individual (i.e., all Seychelles species except *Hypogeophis brevis*) and for a sister group relationship between *Grandisonia larvata* and *G. sechellensis*, but otherwise relationships between the species were variably resolved in the different analyses and generally with only low support. Accepting the rooting of the Seychelles caecilian tree with *Praslinia cooperi* and collapsing *G. larvata* + *G. sechellensis* into a single taxon reduces the remaining interrelationships to a four-taxon problem, for which there are 10 possible clades and 15 distinct rooted trees. Table 6 summarises the support for these 10 clades across different analyses. All 10 possible clades occur across the bootstrap/Bayesian trees but several clades are never supported by more than 50% of the trees from any single analysis. Using the notation A = *G. alternans*; B = *H. brevis*; L = *G. larvata* & *G. sechellensis*; R = *H. rostratus*, the groupings that never receive majority support are AL, AR, ARL, ABR, BR, and BLR (Fig. 2). Two hypotheses, AB and LR, have majority support only in LogDet analyses, highlighting the potential for the moderate to high support for some conflicting hypotheses (e.g. ALP and BL) to be an artefact of base compositional biases in these data. Unsurprisingly, analyses of the smallest dataset (tRNA) yield the smallest maximum support values for any clade.

Figure 2 provides a complementary summary of the frequency of occurrence of all possible 15 rooted trees. Note that only two of the 15 trees (trees 2 and 13) ever form a majority in any of the bootstrap analyses. Overall, the pattern of low to moderate support (that is not sustained across multiple analyses) suggests that the data are simply not sufficient for resolving relationships among these four taxa.

Comparisons of support for full and partial splits across the various analyses (Table 6) provide no indication that instability associated with any specific 'rogue" taxon is obfuscating support for otherwise well-supported partial splits.

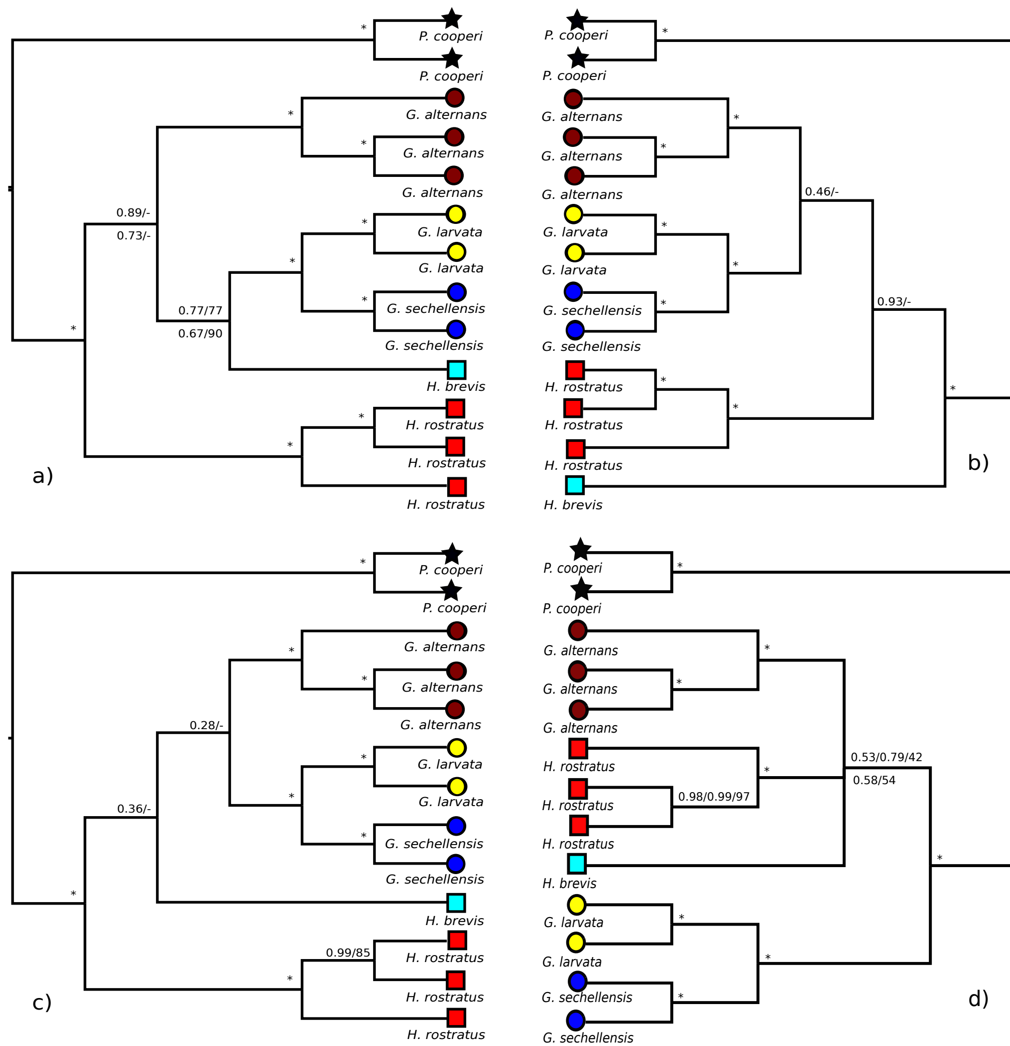

Fig. 1 The four phylogenetic tree topologies inferred from the five data sets. (a) both the complete nucleotide data and the protein-coding nucleotide data (b) rRNA, (c) tRNA (d) amino acids. In (a) numbers above branches are for the complete nucleotide data and below for the protein-coding nucleotides (BI/ML). In (b) and (c) numbers above branches are BI/ML. In (d) values above branches are Bayesian posterior probabilities for the unpartitioned CAT and CATGTR analyses run on PhyloBayes; values below branches are BI/ML support for the gene-partitioned dataset. Maximal support is indicated by a single X and support values below 0.5/50% (BI/ML) are indicated by "-" (or by collapsed branches in the PhyloBayes tree (d)). Symbols at terminals refer to genus: stars = *Praslinia*; squares = *Hypogeophis*; circles = *Grandisonia*. Colours refer to species: black = *P. cooperi*; red = *H. rostratus*; turquoise = *H. brevis*; brown = *G. alternans*; yellow = *G. larvata*; blue = *G. sechellensis*. All trees were rooted with *Praslinia cooperi*.

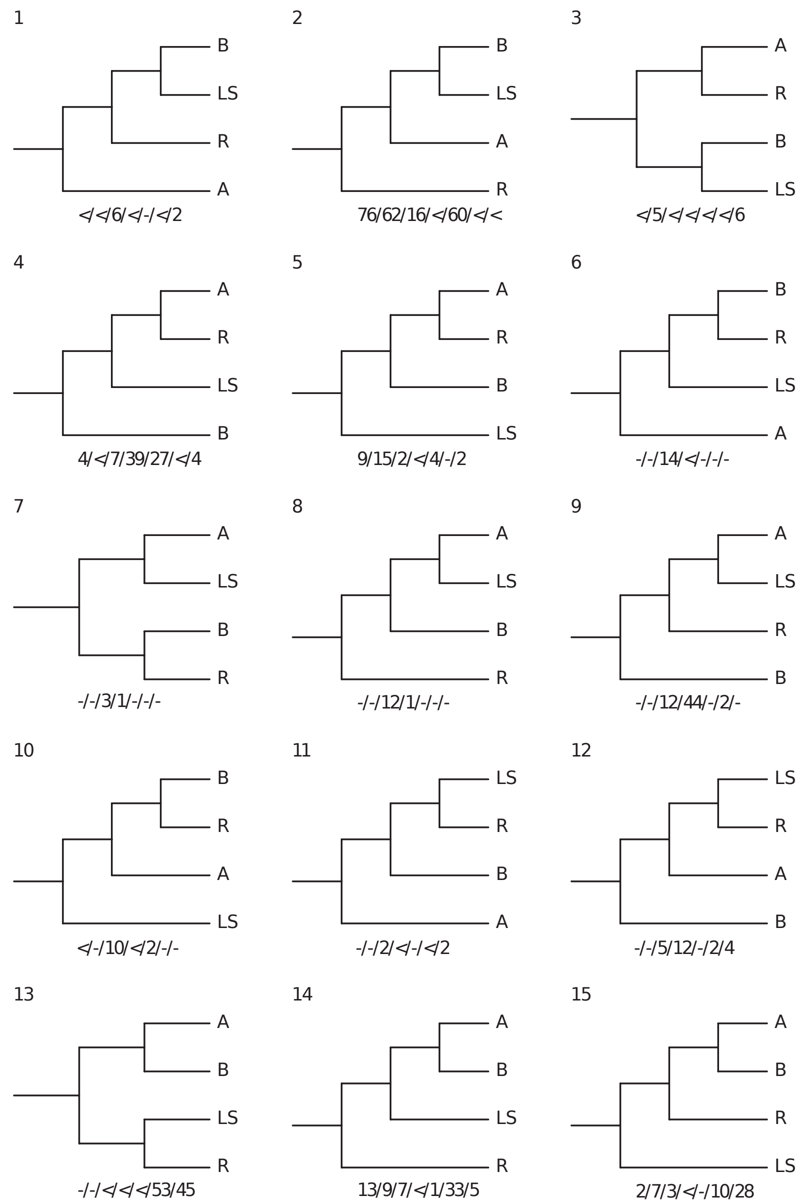

Fig. 2 The fifteen rooted trees for the four taxa used to assess taxon instability and their percentage frequency of occurrence in 1000 Bayesian or Bootstrap (LogDet) trees. Taxa abbreviated as follows: *Grandisonia alternans* (A), *Hypogeophis brevis* (B) *Grandisonia larvata* + *Grandisonia sechellensis* (LS) and *Hypogeophis rostratus* (R). Numbers below trees are support values for: all nucleotide data/ protein-coding / tRNA / rRNA /amino acids/ LogDet for all data / LogDet for protein-coding data. < = less than 1% support, - = zero support.

Table 7. Summary of percentage of support for clades presented in Fig. 2. A = *G. alternans*, B = *H. brevis,* L= *G. larvata* + *G. sechellensis*, R = *H. rostratus*. - indicates zero support. Abbreviations in column 1 are as follows: BI = Bayesian Inference analysis; LD = LogDet analysis; All = complete nucleotide dataset; rRNA = rRNA dataset; tRNA = tRNA dataset; PC = protein coding nucleotide dataset; AA = amino acid dataset.

Analysis	AB	AL	AR	ABL	ABR	ALR	BL	BR	BLR	LR
BI All	15.1	-	8.2	88.9	6.8	3.9	76.4	0.6	0.1	-
LD All	95.3	0.2	1.2	33.4	9.8	3	1.5	-	0.5	55.1
BI rRNA	1,2	46.1	39.5	1,9	1.1	94.3	0.7	2.4	0.8	12,0
BI tRNA	10.2	27,6	9.6	35.6	14.2	24	22.9	26.3	22.2	7.4
BI PC	16.2	-	21	71.2	22.4	0.8	67.9	-	0.5	-
BI AA	1	-	36.4	61.2	11.5	26.7	60.8	2.4	-	-
LD PC	78.4	-	12.1	5.4	30.2	8,5	8.7	-	4.6	52.1

5.4 Discussion

5.4.1 NGS mitogenomics

In our experience, the overall most cost-effective method for obtaining mitochondrial genomes when total time and accuracy were taken into account was the shotgun sequencing approach with the Illumina MiSeq platform (Table 2). Although sequencing costs are much lower for generating complete mitogenomes with long-amplicon, multiplex and Sanger sequencing, it is more time intensive in terms of bench work and sequence handling. The multiplex data provide a much more enriched sample set but they require a large amount of time and, particularly for the Illumina HiSeq data, more computing power to process the data. Our multiplex data were not individually indexed, which increased the time required to reconstruct mitogenomes, and made it impossible to ensure with absolute certainty that all the constituent fragments in each reconstructed mitogenome pertain to a single individual specimen. In our case, we were able to partly address the latter concern because our multiplex datasets included only one sample of each species and because mitogenome sequences of the same specimen and/or conspecifics or close relatives were available as references. Although the Illumina MiSeq is

probably the most expensive method that we used per sample (~$430, in a total sample of six), it is fast for generating mitogenomes in terms of time required for lab work, sequencing and post-sequencing analysis and reconstruction. However, some MiSeq samples lacked high sequence coverage when compared with multiplex sequencing on the Illumina HiSeq. In addition, and because the samples were indexed, our MiSeq approach allowed us to attribute sequenced fragments to the mitogenome of each individual with almost complete certainty (assuming lack of contamination). This shotgun sequencing method also provides data that can be used for other purposes, such as development of anonymous nuclear loci (Lewis *et al.* 2014), future development of microsatellite markers (e.g. Nowak *et al.* 2013) or for SNP identification (e.g. Schwartz *et al.* 2013).

5.4.2 Molecular phylogeny and systematics of Seychelles caecilians

Our analyses suggest that mitogenomic data are simply not sufficient for resolving all relationships among Seychelles caecilians. Additionally, there appears to be some substantial base-composition heterogeneity in the protein-coding genes that might be misleading phylogenetic inferences based on these data. It is noteworthy that the pairing of *Hypogeophis rostratus* and *H. brevis* is almost never supported, and this calls into question this taxonomy proposed by Wilkinson *et al.* (2011). However the inadequacy of the data seems to preclude ruling out anything at this stage other than relationships that contradict the well-supported sister-group relationship between *Grandisonia larvata* and *G. sechellensis* that was found in many previous analyses also (e.g. Gower *et al.* 2011; Hedges *et al*. 1993; Loader *et al*. 2007;

Wilkinson *et al.* 2002, 2003). With additional sampling (e.g., a second individual of *H. brevis*) there is the potential to improve the resolution of Seychelles caecilian phylogeny based on mitogenomes, but it seems more likely that the remaining phylogenetic problems will require additional sequence data from nuclear genes.

CHAPTER 6

ATTEMPTING TO SOLVE THE UNSOLVABLE? PHYLOGENETICS OF AN ANCIENT RADIATION OF CAECILIAN AMPHIBIANS

Abstract

Inferring the phylogenetic relationship among species has become ever more reliant on nuclear loci. However, inferring species trees has only relatively recently become common practice. Many recent studies solely utilise a single species tree method without considering the impact that the method may have on the final topology. The Seychelles caecilian amphibians, an endemic radiation of the Seychelles, have generally proven resistant to phylogenetic resolution in previous molecular studies. By generating and analysing data for two mitochondrial and 13 nuclear loci this chapter attempts to reconstruct the species level relationships of the Seychelles caecilian radiation. Seven different methods utilising Bayesian, maximum likelihood, parsimony and distance based approaches were implemented. A considerable lack of congruence was observed across the different methods and only two produced identical topologies, a worrying scenario considering that only eight species are examined and many other studies examine relationships between much larger numbers of taxa. However, *Praslinia cooperi* is considered to be the sister to the other, “core taxa” and in most analyses there was support for three main core taxa clades: *Grandisonia larvata* + *G. sechellensis*, *G. alternans* + *Hypogeophis rostratus*, and *H. brevis* plus the two undescribed dwarf species. The lack of compelling resolution is thus on relationships among these three clades.

6.1 Introduction

The shift from gene-tree to species-tree phylogenetic inference to uncover relationships among taxa has grown considerably in recent years. This shift from gene- to species-trees is important when investigating species-level phylogenies because incomplete lineage sorting and other discordance among gene trees (Degnan & Rosenberg 2006; Rosenberg 2013) may paint a false picture of actual species relationships when concatenating unlinked loci (e.g. Kubatko and Degnan, 2007).

Methods to infer species trees vary greatly and now include simpler pseudolikelihood methods (Liu *et al.* 2010; Jewett & Rosenberg 2012), maximum likelihood (Kubatko *et al.* 2009) and more complex Bayesian approaches (Liu 2008; Heled & Drummond 2010). The less complex models have generally received less recognition in the literature, perhaps because they are more involved and require user inferred gene trees for each locus prior to their implementation e.g. MP-EST (Liu *et al.* 2010), STAR, STEAC (Liu *et al.* 2009), and NJst (Liu & Yu 2011). Species trees that implement the multispecies coalescent have been found to outperform other methods (Heled & Drummond 2010; Leaché & Rannala 2011). However, with the advent of large datasets generated from next-generation sequencing it is not feasible to compute species trees using the more complex, commonly used Bayesian methods. One exception is the recently developed program SNAPP (Bryant *et al.* 2012), which uses a full Bayesian MCMC sampler but its application does not stretch to full loci; only being applicable for SNP and AFLP data.

The most widely and commonly used species tree method is the Bayesian multispecies coalescent method *BEAST (Heled & Drummond 2010) although

it is probably wise to test different species tree methods to look for congruence (e.g. Camargo et al., 2012; Jockusch et al., 2015). *BEAST has generally been shown to outperform other species tree methods and relaxes many of the assumptions employed by these (Heled and Drummond, 2010: but see Jockusch et al., 2015).

One relatively overlooked method in species-level phylogenetics is combining multiple allelic information by standardising distance matrices between unlinked markers as implemented in POFAD (Joly & Bruneau 2006). This approach is seemingly robust at inferring relationships among hybrid individuals and is ideal for closely related species (Joly & Bruneau 2006; Wallace *et al.* 2009; Leaché *et al.* 2009), in addition to performing well at higher taxonomic levels (Yu *et al.* 2011b; a).

The mostly fossorial caecilian amphibians (Gymnophiona) of the Seychelles form a monophyletic group (Gower et al., 2011; Hedges et al., 1993; Loader et al., 2007; Pyron and Wiens, 2011; Pyron, 2014; San Mauro et al., 2012, 2014; Wilkinson et al., 2002, 2003; Zhang and Wake, 2009) of six described (Nussbaum 1984a) and two undescribed species. The separation of the Seychelles islands has prevented contact or gene flow between the caecilians here and their closest living relatives in the Western Ghats, India for at least 65 Ma (e.g. Armitage et al., 2011; Collier et al., 2008; Gunnell et al., 2003). Most Seychelles caecilian species, except for the two undescribed species, can be found in sympatry (Nussbaum, 1984; *unpub. data*) on the largest island of Mahé and the high degree of potential habitat overlap suggests it is possible that hybridisation may have occurred throughout the speciation process.

Within the Seychelles radiation *Praslinia cooperi* is the sister to all other species (Gower et al., 2011; Loader et al., 2007; San Mauro et al., 2012, 2014; Wilkinson et al., 2002, 2003; Zhang & Wake, 2009). Two recent studies have failed to recover this relationship (Pyron & Wiens 2011; Pyron 2014), which is likely attributed to considerable amounts of missing data in their analyses and or unnecessarily distant outgroup selection (M. Wilkinson *pers. comm.*). The relationship of the non-*Praslinia* (here "core") Seychelles species has remained unresolved in molecular phylogenetic analyses except for the sister species relationship between *Grandisonia larvata* and *G. sechellensis* (Chapter 5; Gower et al., 2011; Loader et al., 2007; San Mauro et al., 2014; Wilkinson et al., 2002, 2003). One species (*Hypogeophis brevis*) has recently been transferred from the genus *Grandisonia* based on molecular analysis (Gower *et al.* 2011) and morphology (see Wilkinson et al., 2011), to the formerly monotypic *Hypogeophis*, although some studies have failed to support this relationship (Loader et al., 2007; Wilkinson et al., 2002, 2003). The position of *G. alternans* within the core group's phylogeny remains unresolved.

In this chapter data are generated for two mitochondrial and 13 unlinked nuclear genes, a combination of protein-coding, non-coding and anonymous nuclear loci. These data are analysed to attempt to resolve the species-level phylogeny of the Seychelles caecilians. Despite substantially increased taxon and genetic sampling and use of multiple methods of phylogenetic inference, a compelling, full resolution was not achieved. I recommend synonymising *Grandisonia* with *Hypogeophis* to remove the distinct possibility that one or both are non-monophyletic.

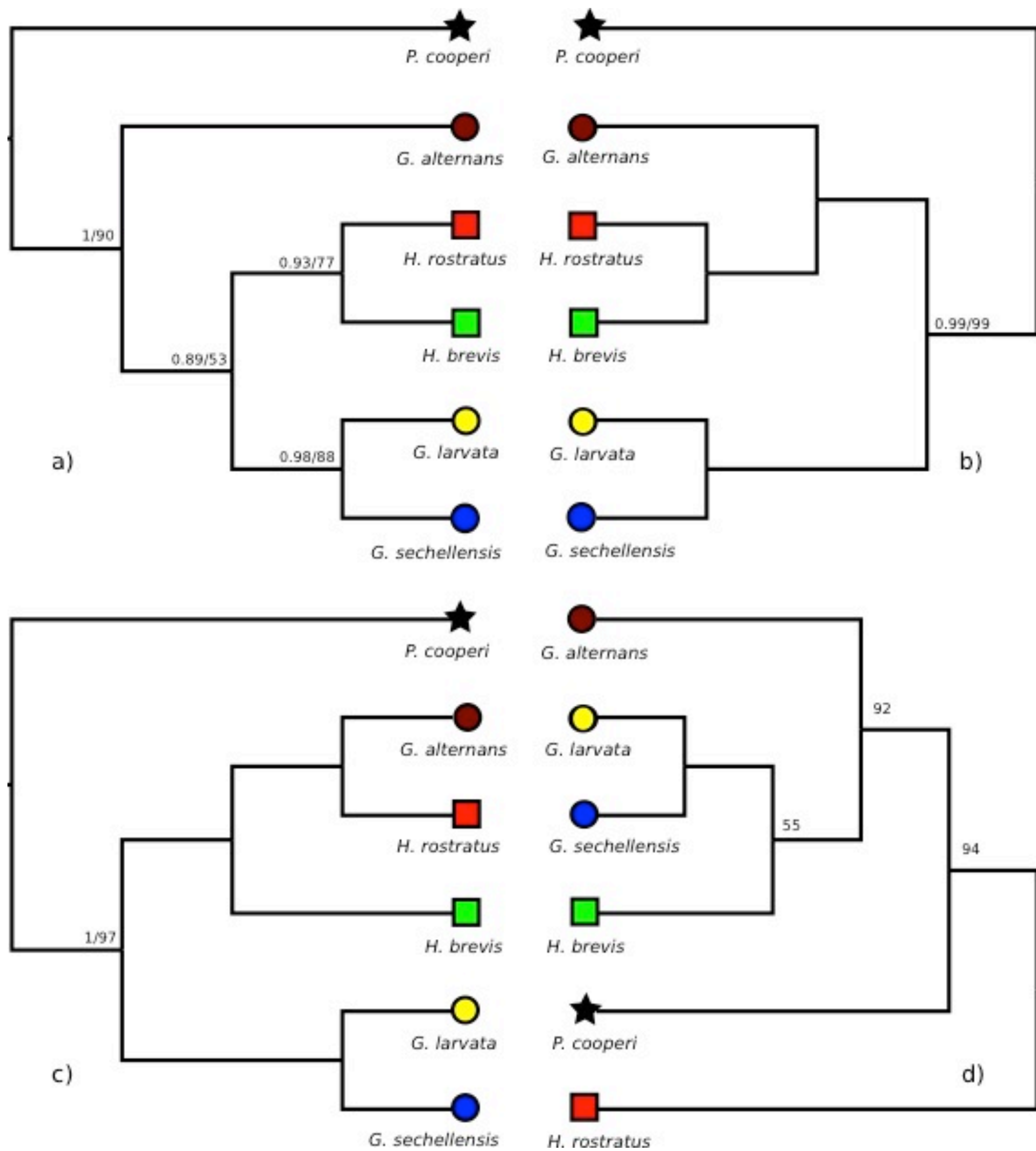

Fig. 1 Phylogenetic trees showing relationships for all Seychelles caecilian species inferred from previously published analyses of molecular data. Only support >50% bootstrap and >0.5 Bayesian posterior probabilities are shown. Symbols at terminals refer to genus: stars = *Praslinia*; squares = *Hypogeophis*; circles = *Grandisonia*. Colours refer to species: black = *P. cooperi*; red = *H. rostratus*; green = *H. brevis*; brown = *G. alternans*; yellow = *G. larvata*; blue = *G. sechellensis*. Phylogenetic tree topologies are as follows: a) Gower et al. (2011); numbers on branches are BI/ML of analyses of protein coding codon positions and rRNA analyses; b) Loader et al. (2007) and Wilkinson et al. (2002). The topology from Wilkinson et al. (2002) had no support values >50%. Numbers on branches are from Loader et al.(2007) and refer to BI/ML; c) Wilkinson et al. (2003); numbers on branches are BI/ML; d) Pyron et al. (2011); numbers on branches are ML bootstrap values.

6.2 Methods

6.2.1 Taxon sampling and molecular markers

Samples of Seychelles caecilians were collected from across most species' ranges over a total of nine out of 10 occupied islands between 1989 – 1991, and 2013 – 2014 ((*Grandisonia alternans*: Mahé = 7, Silhouette = 24, Praslin = 4, La Digue = 6, Fregate = 18; *G. larvata*: Mahé = 2, Silhouette = 13, Praslin = 4, La Digue = 14, Ste Anne = 1; *G. sechellensis*: Mahé = 18, Silhouette = 21, Praslin = 3; *Hypogeophis brevis*: Mahé = 14; *H.* cf. *brevis* Praslin: Praslin = 16; *H.* cf. *brevis* CR: Mahé = 2; *H. rostratus*: Mahé = 4, Silhouette = 6, Praslin = 5, La Digue = 3, Fregate = 2, Ste Anne = 2, Curieuse = 2, Cerf = 2; *Praslinia cooperi*: Mahé = 6, Silhouette = 6) see Appendix 5)). Samples were collected either destructively from liver, heart and muscle or non-destructively following Maddock et al. (2014), and stored in 100% ethanol or snap frozen at -80°C.

Complete genomic DNA was extracted using either the Qiagen DNeasy Blood and Tissue Kit or a standard phenol-chloroform extraction protocol (Sambrook *et al.* 1989). Qiagen kit extraction followed manufacturer guidelines with the exception of the final suspension of the extracted DNA being stored in 2x100µl buffer AE.

Standard PCR was used to amplify DNA fragments from two mitochondrial and 13 nuclear loci. The mtDNA sequences consisted of the protein-coding cytochrome *b* (*cytb*) and non-coding 16s rRNA (*16s*) genes. The nuclear data comprised five protein-coding loci: recombination-activating gene 1 (*rag1*), pro-opiomelanocortin (*pomc*), brain-derived neurotrophic factor (*bdnf*), histone H3 (*h3*), and SLC8A1 solute carrier family 8 (*slc8a1*); one non-coding exon: rhodopsin (*rho*); and seven non-coding anonymous nuclear loci

(ANL): *alt15, alt23, brev2, brev5, rost1, rost5* and *sech5* (see Lewis et al., 2014). See table 1 for primer information. PCR reaction volume was 25µl and consisted of 12.5µl of MyTaq Mix x 2, 9.5µl of ddH_2O, 1µl of forward and reverse primers, and 1µl of template DNA. Cycling conditions for ANL followed (Lewis *et al.* 2014). All other amplification conditions were as follows: 2:00 at 95°C; 40 x [0:30 at 95°C; 0:30 at 46°C for *h3*, 0:30 at 48°C for *cytb*, 0:30 at 55°C for *rag1* and rhod*,* 58°C for *bdnf* and *pomc*; 0:30°C at 72°C] or 35 x [0:30 at 95°C; 0:30 at 46°C for *slc8a1*; 0:30°C at 72°C]; and a final extension of 72°C for 4:00

Table 1. Primers used in this chapter for PCR and sequencing.

Gene	Primer name	Primer (5' – 3')
cytb	L14724[c]	CGAAGCTTGATATGAAAAACCATCGTTG
	CB3H[d]	GGCAAATAGGAAGTATCATTCTG
16s	16SA-L[e]	CGCCTGTTTATCAAAAACAT
	16SB-H[e]	CCGGTCTGAACTCAGATCACGT
pomc	POMC_DRV_F1[a]	ATATGTCATGASCCAYTTYCGCTGGAA
	POMV_DRV_R1[a]	GGCRTTYTTGAAWAGAGTCATTAGWGG
bdnf	BDNF_DRV_F1[a]	ACCATCCTTTTCCTKACTATGG
	BDNF_DRV_R1[a]	CTATCTTCCCCTTTTAATGGTC
rhodopsin	Cae_Rod-Ex1.F1[b]	TTTYTATRTTCCCATGTCAAAYAAGACYGG
	Cae_Rod-Ex2.R1[b]	GGCCATVATCCAVGTAARYAMGACHCCCAT
h3	H3F[f]	ATGGCTCGTACCAAGCAGACVGC
	H3R[f]	ATATCCTTRGGCATRATRGTGAC
slc8a1	Amp-NCX1.F[g]	ATTCTTCTSTCTGTBATTGARGT
	Amp-NCX1.R[g]	AGRAAGTTCTCRTCTTCTTCRAA
rag1	Amp-RAG1.F[h]	AGCTGCAGYCARTACCAYAARATGTA
	Amp-RAG1.R1[h]	AACTCAGCTGCATTKCCAATRTCACA
	Amp-RAG1.F1[h]	ACAGGATATGATGARAAGCTTGT
	Amp-RAG1.R[h]	TTRGATGTGTAGAGCCAGTGGTGYTT
alt15	alt15_F[i]	GCCTTGCATCCCCTAATACA
	alt15_R[i]	GCACACACTGTCGGCTTAAA
alt23	alt23_F[i]	TCCATAGGAAGGGAGCAAGA
	alt23_R[i]	CTGCCCGCTTTCTTTGTAAC
brev2	brev2_F[i]	TAGAAGCCGAGGGTTATTGG
	brev2_R[i]	GAAGAGAAGGTGGGACAGGA
brev5	brev5_F[i]	CATCAGGTCATTGGCGTTTA
	brev5_R[i]	GAGTGCAGGGACCAAATACC
sech5	sech5_F[i]	GCAGCTCTTTCTGTGCCTTT
	sech5_R[i]	GTCTGCCATTGCTGTATGGA
rost1	*rost1*_F[i]	TCTGGAATTGGCCTTGTGTT
	*rost1*_R[i]	CCCACATTCTTCCTCCCTCT
rost5	rost5_F[i]	TGTCAACTGCCCTCTGTGTC
	rost5_R[i]	AAATTCACAGGCCAAACAGG

[a] Vieites *et al*. (2007), [b] S. Mohun (unpublished data), [c] Irwin *et al.* (1991), [d] Moritz *et al.* (1992), [e] Palumbi et al. (1991), [f] Frost *et al.* (2006), [g] D. San Mauro (unpublished data), [h] San Mauro *et al.* (2004), [I] Lewis *et al.* (2014).

6.2.2 Sequence editing and alignment

Sequences were checked and edited using Geneious v.6.1.4 (Biomatters). After initial de-novo assembly of paired reads sequences were trimmed and manually edited. Sequences were then aligned using MUSCLE (Edgar 2004a)

with default settings in Geneious. The *16s* alignment was parsed through GBlocks (Castresana 2000) to remove ambiguously aligned regions. The parameters were set to allow for gaps in the dataset and with less strict flanking positions.

6.2.3 Mitochondrial data analysis

Bayesian inference (BI) and maximum likelihood (ML) trees were constructed for a concatenated dataset of *16s* and *cytb* using MrBayes v.3.2.2 (Ronquist *et al.* 2012) and RaxML v.8.0.24 (Stamatakis 2014), respectively. Partitioning strategy and best-fit models of nucleotide evolution were selected based on results from PartitionFinder v.1.1.1 (Lanfear et al., 2012). The Indian caecilians *Gegeneophis danieli* (GenBank accessions: HQ444080 *cytb*; HQ443991 *16s*) and *Indotyphlus battersbyi* (GenBank accessions: HQ444104 *cytb*; HQ444016 *16s*) were used as outgroups for both sets of analyses based on the results of Gower et al. (2011). MrBayes was run for 10^6 generations and sampled every 10,000 generations with one cold and three heated chains at default temperatures. Chains were checked for convergence using Tracer v1.5 (Drummond & Rambaut 2007) and the first 10% of trees were discarded as burn-in. Convergence was also checked using AWTY (Nylander *et al.* 2008). RaxML analyses were employed with 500 bootstrap iterations using the default option (Stamatakis 2006) of the GTR+CAT substitution model for each partition followed by GTR+G inference for the final tree topology. Genetic distance data for each mitochondrial locus was estimated under the Kimura two-parameter model (Kimura 1980) using MEGA v.6.0.6 (Tamura *et al.* 2013).

Mitochondrial data were not included in any multi-locus analyses because it has been found that the inclusion of mtDNA can significantly impact topologies and that large numbers of loci are required to counteract this impact (Jockusch *et al.* 2015).

6.2.4 Nuclear data analysis

Nuclear locus alignments were run through the program PHASE v.2.1.1 (Stephens *et al.* 2001; Stephens & Scheet 2005) to infer heterozygous positions and to estimate nucleotide bases for shorter sequences. Each dataset was run three times with a random starting seed selected by random.org. Files for PHASE were prepared using seqPHASE (Flot 2010). Heterozygous positions were accepted as true alleles if they had a probability >0.7 based on the results of Harrigan et al. (2008). The PHASE files were used in all subsequent analyses. Partitioning strategy and best-fit models for each nuclear locus were selected based on results from PartitionFinder v.1.1.1 (Lanfear et al., 2012) and used in all analyses where it is possible to implement multiple different models of nucleotide evolution.

A concatenated parsimony analysis for all loci was conducted using PAUPX V. 4.0a142 (Swofford 2002). A single *Grandisonia sechellensis* sample (SM164) was removed because it had data for only one locus. Indels were treated as a fifth character state because this approach has been found to outperform treating them as missing data (e.g. Ogden and Rosenberg, 2007). A replicate analysis was carried out in which indels were treated as missing data, support was less than when indels were treated as a fifth character, and is not shown here. . 100,000 trees were retained out of a

possible 4,046,344,299 trees sampled using a Heuristic search. A majority-rule consensus tree was created for the retained trees.

A standardised distance matrix for all 13 nuclear loci was generated using POFAD v.1.03 (Joly & Bruneau 2006). POFAD creates a single distance matrix, taking into account, with equal weighting, matrices from multiple loci. Genetic distance data for each locus under the Kimura two-parameter model (Kimura 1980) were obtained using MEGA v.6.0.6 (Tamura *et al.* 2013). Neighbour-net, UPGMA and neighbour-joining trees were constructed for the standardised distance matrix using SplitsTree v.4.13.1 (Huson & Bryant 2006).

Many species-tree algorithms require gene trees to be used as input. Indels are potentially phylogenetically informative (e.g. Egan and Crandall, 2008; Ogden and Rosenberg, 2007; Redelings and Suchard, 2007; Simmons and Ochoterena, 2000) but traditional maximum likelihood algorithms treat them as missing data. From manual inspection of the data it seems likely that indels might be informative in this dataset. Gene trees were estimated using RaxML v.8.0.24 (Stamatakis 2014) and GARLI v.2.0 (Zwickl 2006). GARLI treats indels as a fifth character state and allows the implementation of more complex evolutionary models than does RaxML. For both programs all loci, regardless of whether or not they were protein coding, were treated as a single partition to reduce complexity and were not rooted in analyses (following software restrictions for ASTRAL). Models implemented in GARLI that were used were those identified as best fit by PartitionFinder. RaxML analyses were run for 500 and GARLI for 50 bootstrap iterations, implementing the default GTR+CAT substitution model for each tree followed by GTR+G inference model to construct the final tree topology.

Species trees were constructed using ASTRAL-II (Mirarab *et al.* 2014), on bootstrapped unrooted nuclear gene trees. Four separate analyses for each of the RaxML and GARLI trees were run: 1) all loci; 2) loci with sequence data for all species; 3) protein-coding loci; and 4) non-coding loci. ASTRAL was designed to construct species trees based on genomic data with hundreds of loci and taxa; however, using the exact option (-x) fewer taxa can be used. Each analysis was implemented using 100 bootstraps and a majority-rule consensus tree was generated in Geneious.

The multispecies coalescent species tree *BEAST (Heled & Drummond 2010) approach implemented in BEAST v.2.1.3 (Bouckaert *et al.* 2014) was used to reconstruct Bayesian inference species trees. *BEAST does not require an input of gene trees but each locus requires some sequence data for each species, therefore only loci for which there were sequence data for all species were used in the analyses. Following preliminary runs, the data supported a constant evolutionary rate for all loci except *rag1*, for which an uncorrelated relaxed clock was implemented; a strict clock was used for all other loci. Analyses were run for 2×10^8 MCMC chains with sampling every 10,000 generations. Convergence of all parameters was assessed using Tracer v.1.5 (Rambaut & Drummond 2009) and the first 10% of trees were discarded conservatively as burn-in.

To investigate the number of topological differences between trees the program KTreeDist (Soria-Carrasco *et al.* 2007) was used to estimate Robinson-Foulds (symmetric) distances.

6.3 Results

Table 2 Sequence information for each locus used in this study. Columns refer to: best-fit substitution models (cp = codon position) used in analyses where it is possible to specify substitution models; number of nucleotides for each locus; PI = parsimony informative sites; VI = variable sites; π = nucleotide diversity; D = Tajima's *D*.

Locus	Models	Nucleotides	PI	VI	π	D
cytb		810	298	327	0.107	1.194
16s		579	128	154	0.057	0.744
16s, cytb (cp1)	GTR+I+G					
cytb (cp2)	GTR+I+G					
cytb (cp3)	GTR+G					
pomc	GTR+I+G	509	46	48	0.021	0.098
bdnf	HKY+I	713	20	21	0.006	-0.294
rhodopsin	HKY+I+G	854	64	81	0.015	-1.333
h3	GTR+I+G	332	16	24	0.01	-1.578
slc8a1	GTR+I+G	844	30	40	0.009	-1.08
rag1	GTR+I+G	1453	59	89	0.005	-2.415
alt15	GTR+I	280	30	30	0.023	-0.041
alt23	HKY+I	288	45	48	0.033	-0.924
brev2	HKY+I+G	198	23	27	0.031	-0.38
brev5	HKY	298	42	45	0.028	-0.505
sech5	HKY+G	252	15	21	0.017	-0.625
rost1	GTR+I	289	22	24	0.02	-0.337
rost5	HKY+I	304	29	32	0.024	0.146

6.3.1 Mitochondrial phylogeny

The maximum likelihood (ML) and Bayesian inference (BI) trees conflict with respect to relationships among core species (Fig. 2); *P. cooperi* is supported as being the sister to all core species. The only relationships that are strongly supported in both analyses are clades comprising *Grandisonia larvata* + *G. sechellensis*, and *H. brevis* + the undescribed dwarf (*brevis*-like) species from Congo Rouge on Mahé. The monophyly of all dwarf species (*H. brevis*, the undescribed species from Congo Rouge, and the undescribed species from Praslin) was not recovered in the ML or BI majority-rule trees. The non-monophyly of *Hypogeophis* and *Grandisonia* (sensu Wilkinson et al. 2011)

could not be rejected. There was moderate support for a clade comprising *G. alternans, H. rostratus, H. brevis* and the undescribed dwarf species from Congo Rouge in the BI analysis (0.76).

Fig. 2 Phylogenetic trees inferred for the concatenated mitochondrial *cytb* and *16s* gene sequences: (a) Bayesian inference (BI) tree inferred from MrBayes; and (b) maximum likelihood (ML) tree inferred from RaxML. The Praslin and Congo Rouge labels refer to the localities of the two undescribed dwarf species. Numbers above branches are Bayesian posterior probabilities (a) and bootstrap support (b). Scale represents substitutions per site.

6.3.2 Gene trees and concatenated nuclear phylogeny

Topological analysis of individual gene trees with both GARLI and RaxML failed to reject monophyly of all species for all loci (see supplementary material). However, support was generally low in individual gene trees and therefore little inference can be made regarding species relationships based on these alone. For most loci all but a single specimen were monophyletic for all species. The most obvious outlier was specimen RAN31310 (*Praslinia cooperi*) which was not sister to conspecifics in analyses of *rag1*, *rhod*, *brev5* or *sech5*.

Concatenated parsimony analysis yielded a maximally supported topology for all species-level relationships (Fig. 3). *Praslinia cooperi* is sister to all other Seychelles caecilians. A clade comprising *Hypogeophis brevis*, the undescribed dwarf species from Congo Rouge, and the undescribed dwarf species from Praslin is sister to a *Grandisonia larvata* + *G. sechellensis* clade. *Hypogeophis* cf. *brevis* Praslin is sister to *H. brevis* sensu stricto and *H.* cf. *brevis* CR. *Grandisonia alternans* and *H. rostratus* form a clade, which is sister to the *larvata-sechellensis* + *H. brevis* group.

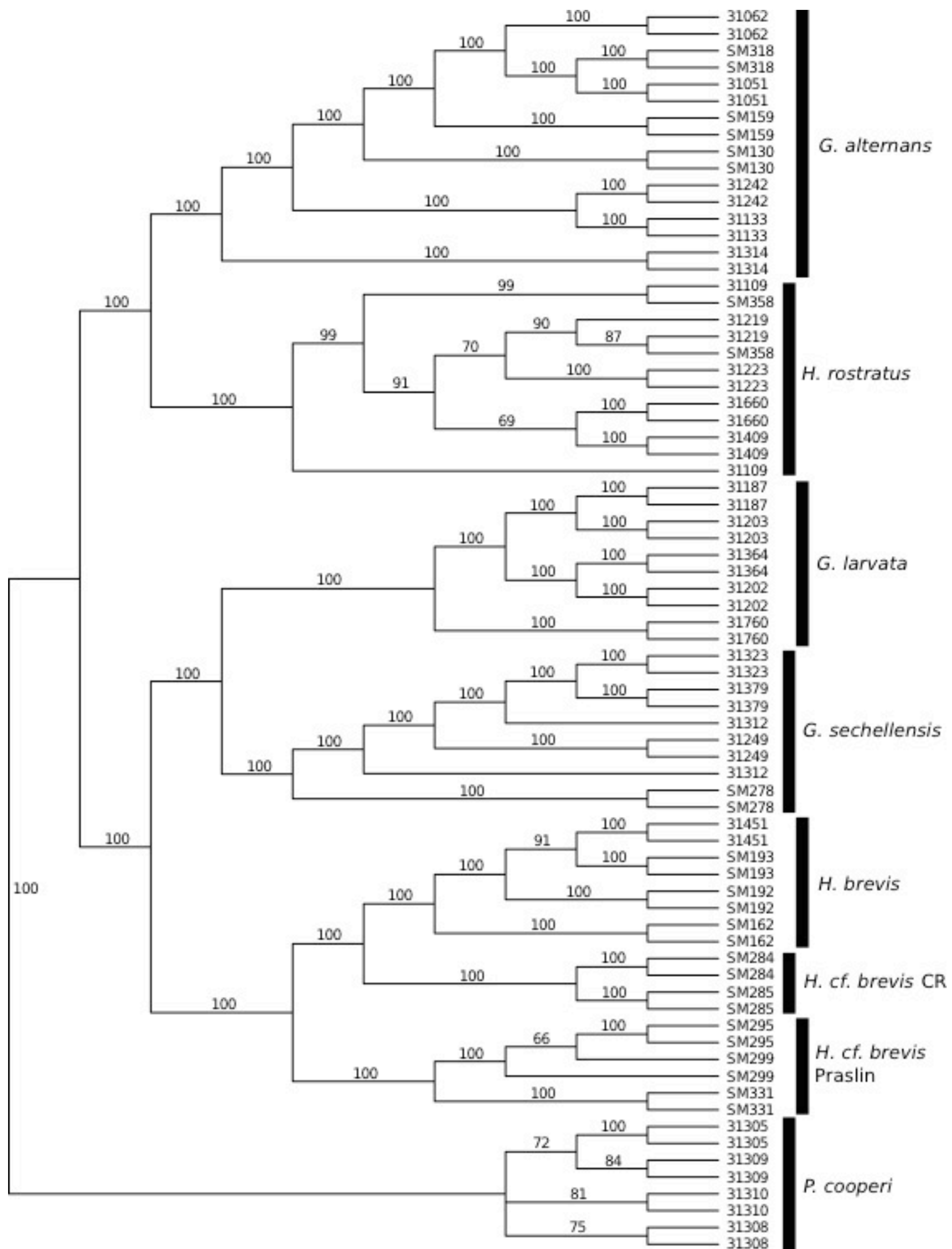

Fig. 3 Majority-rule consensus parsimony tree for all nuclear loci from analysis in which indels are treated as a fifth character state. Numbers above branches represent bootstrap support values. Support values < 50 are collapsed into polytomies.

Neighbour-joining and UPGMA phylogenies for the standardised distance matrices provided identical topologies (Fig. 4). Both analyses recover *P. cooperi* as sister to the core Seychelles taxa. All three of the dwarf species form a clade, with the two dwarf species from Mahé (*H. brevis* and the *H.* cf. *brevis* CR) forming a monophyletic group. The two most widespread caecilians, *G. alternans* + *H. rostratus* form a clade and *G. larvata* + *G. sechellensis* form a clade; these two clades form a larger monophyletic group, which is sister to the dwarf species group. The neighbor-net algorithm (Fig. 4) supports the same clustering of groups as the neighbor-joining and UPGMA phylogenies.

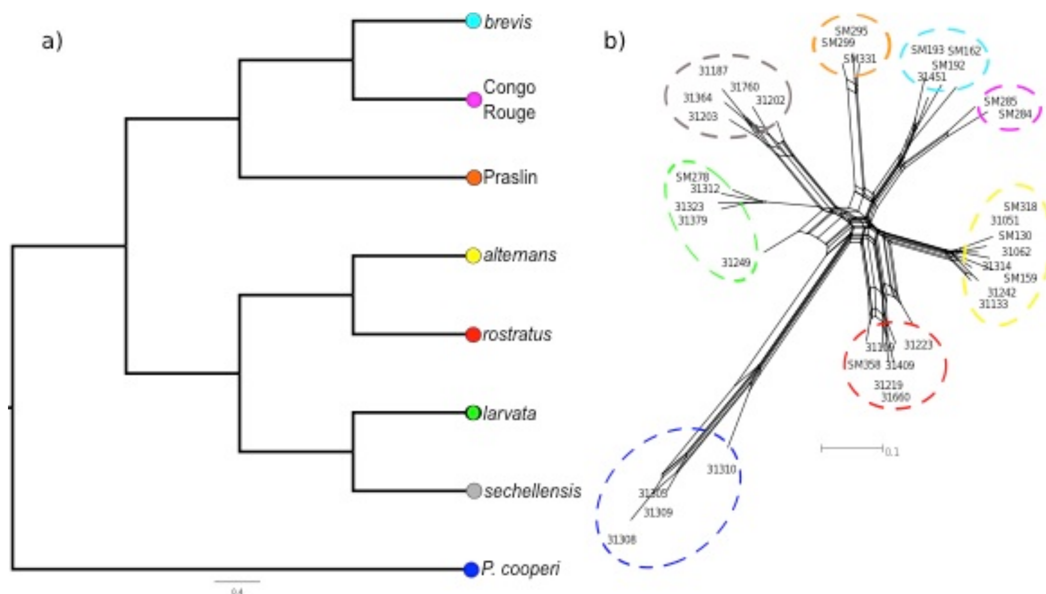

Fig. 4 Nuclear distance based phylogenetic relationships inferred using POFAD: a) neighbour-joining tree, b) SplitsTree median joining network. Coloured ellipses on network correspond to those describing species in the neighbour-joining tree.

6.3.3 Species trees

Bayesian species tree analyses using *BEAST recovered conflicting topologies among the different dataset analyses (Fig. 5). The complete dataset generally yielded the highest posterior probabilities (bpp) for relationships among the core taxa; *P. cooperi* was recovered as the sister to the other species. The only exception was the *G. larvata* + *G. sechellensis* clade, which has a bpp of 0.71 for the complete dataset and 0.89 for the protein-coding dataset. The *G. alternans* + *H. rostratus* clade is strongly supported for the all species dataset (bpp 0.96). The monophyly of the three dwarf species was not strongly supported, however *H. brevis* sensu stricto and *H.* cf. *brevis* CR did form a clade. The *G. alternans* + *H. rostratus* clade received moderate support (bpp 0.85) as the sister group to *H. brevis* sensu stricto, *H.* cf. *brevis* CR, *H.* cf. *brevis* Praslin, *G. larvata* and *G. sechellensis* to but not in the protein-coding dataset (bpp 0.34).

The only relationships recovered in the non-coding species tree inferred with *BEAST that are fully compatible with that of the complete and protein-coding datasets are the sister relationship between *G. alternans* + *H. rostratus* (bpp 0.98), and between the undescribed dwarf species from Congo Rouge + *H. brevis* (bpp 0.94). The dwarf species from Praslin forms the sister to the dwarves from Mahé (bpp 0.87). All other relationships are weakly supported except for a clade comprising *P. cooperi* and *G. larvata*.

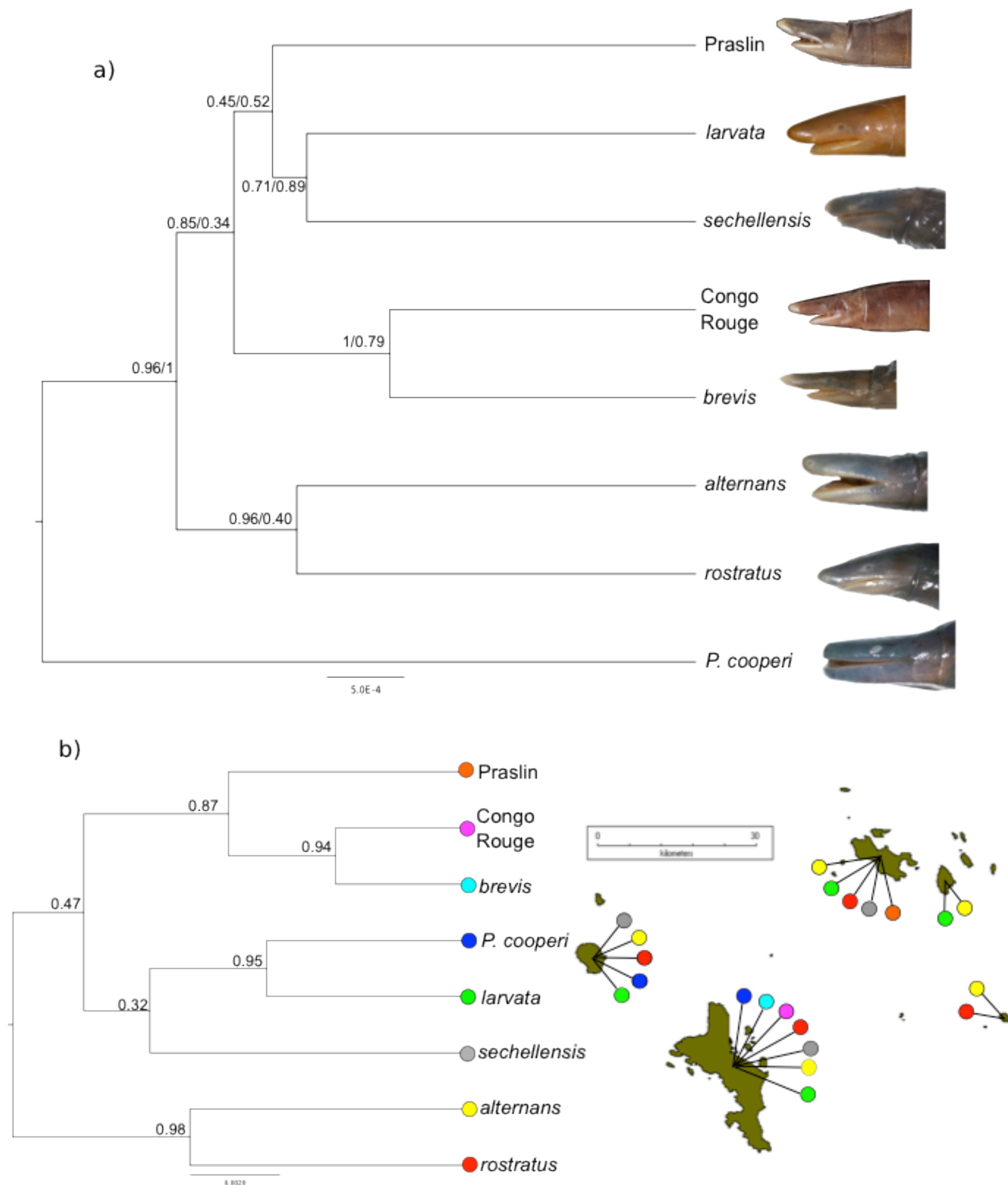

Fig. 5 Bayesian species trees of the relationships among the Seychelles caecilian species inferredusing *BEAST: a) topology for the species where all loci were successfully amplified and protein-coding datasets; b) topology for the non-coding dataset, colours refer to those used in Fig. 5c; c) island sampling localities for the species used in this chapter, coloured circles refer to species in Fig. 5b. Numbers on branches refer to Bayesian posterior probabilities (bpp). In 5a the bpp scores are indicated separately for loci present in all species / protein coding datasets.

The different datasets analysed with ASTRAL (all loci, all loci with sequence data for each species, protein-coding loci, and non-coding loci) each yielded differing topologies (Fig. 6). All analyses constructed using either RaxML or GARLI gene trees for the datasets yield the same topologies except for when all loci were used. Because ASTRAL uses unrooted gene trees to build species trees, the trees were rooted with *P. cooperi* post analysis. Each analysis recovered the *G. larvata* + *G. sechellensis* clade with generally high support (the exceptions being for the non-coding dataset with supports of 78 and 73 for GARLI and RaxML analyses, respectively; and for the all-locus analysis using RaxML with a support of 73). The *H. brevis* + Congo Rouge dwarf clade receive strong support in all analyses. All analyses support the sister relationship between the undescribed dwarf species from Praslin and the Mahé dwarf species but with varying levels of low support. A *Grandisonia alternans* + *H. rostratus* clade receives maximal support in all analyses. Although the analyses recovered *G. alternans* + *H. rostratus, G. larvata* + *G. sechellensis* and a *H. brevis* group clade, relationships among these clades are not resolved.

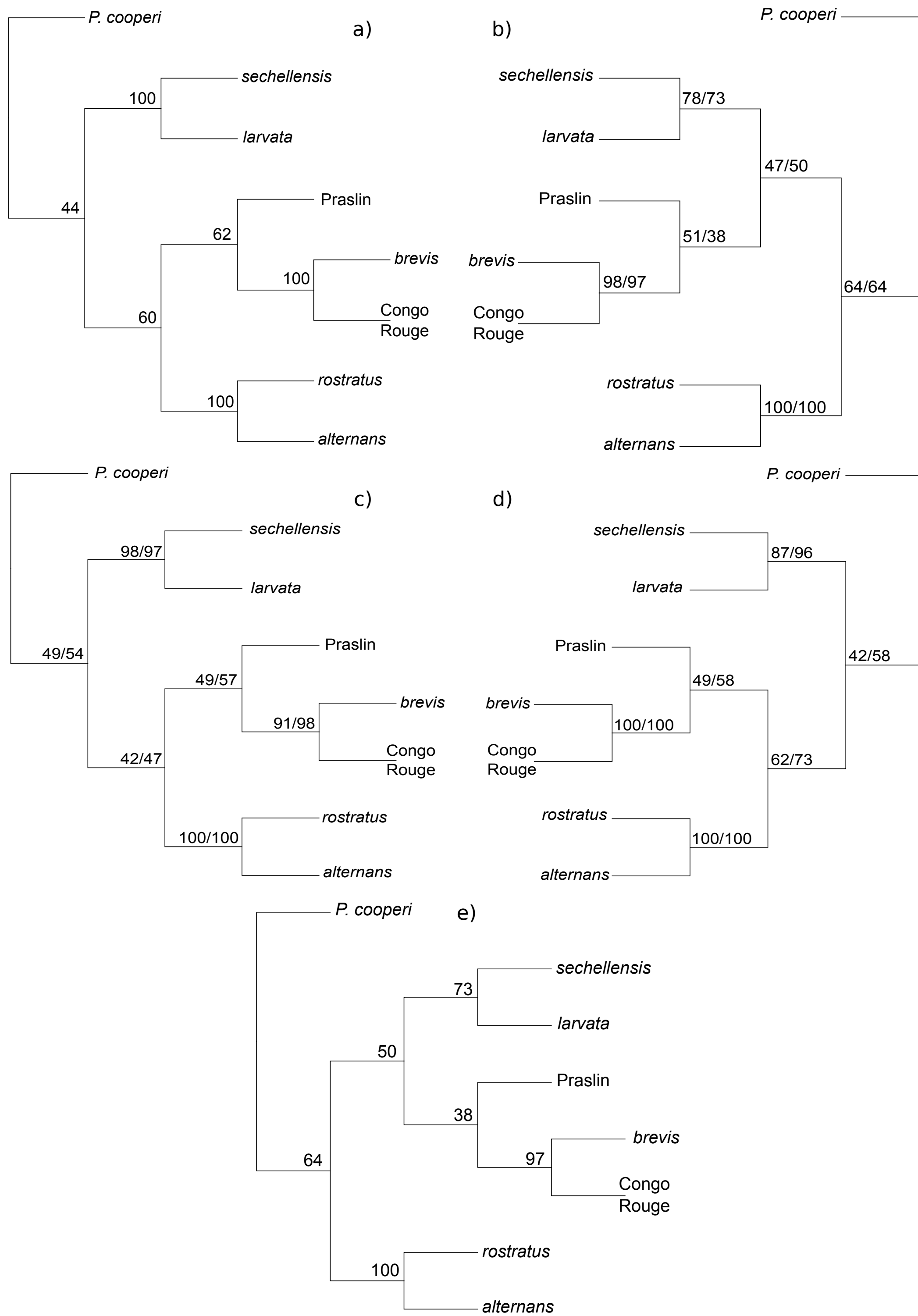

Fig. 6 The five species tree topologies inferred from the ASTRAL likelihood approach. Unless stated, the topologies for both the GARLI and RaxML input trees were identical. Trees shown are those yielded by analyses of: a) all loci using GARLI input trees, b) non-coding dataset, c) protein-coding dataset; d) a dataset where all loci were successfully amplified for each species e) all loci, using RaxML. When two support values appear above branches they refer to results from the GARLI / RaxML input analyses.

Table 3 Robinson Foulds distances between phylogenetic trees inferred using different methods. If matching topologies were recovered using the same method but a different dataset then these are grouped together in the table. Abbreviations refer to: CD = complete dataset, PC = protein coding locus dataset, NC = non-coding locus dataset, FS = complete dataset for all species. If a (G) is present in ASTRAL analyses directly following abbreviation then it refers to gene trees reconstructed using GARLI and if an (R) is present then gene trees were inferred using RaxML; if neither are present both RaxML and GARLI input gene trees produced identical species tree topologies.

	mtDNA mrBayes	mtDNA RaxML	*BEAST FS, PC	*BEAST NC	POFAD	PAUPX	ASTRAL CD(G), PC, FS	ASTRAL NC(R)
mtDNA mrBayes	0							
mtDNA RaxML	4	0						
*BEAST FS, PC	6	4	0					
*BEAST NC	10	10	8	0				
POFAD	6	6	4	6	0			
PAUPX	6	6	2	6	2	0		
ASTRAL CD(G), PC, FS	6	6	4	6	2	2	0	
ASTRAL NC(R)	6	6	2	6	2	0	2	0

6.4 Discussion

6.4.1 Comparison of phylogenetic approaches

The analyses conducted here generally yield conflicting topologies except for the nDNA concatenated parsimony analysis and the analysis of the nDNA non-coding dataset using the species tree method ASTRAL. This is a worrying result considering that a total of 13 different analyses were employed and many recent phylogenetic studies solely run a single analysis to infer species level relationships (e.g. Barlow et al., 2013; Blanco-Pastor et al., 2012; Maddock et al., 2014a; Rocha et al., 2013; Satler et al., 2011; Wiens et al., 2012; Wood et al., 2011). The lack of compelling resolution in the Seychelles caecilian case might have been overlooked if only a single approach had been used. Despite a large number of studies moving towards carrying out species tree analyses, there is still a black box issue regarding the impacts of different analytical approaches. The majority of studies employing small to moderate numbers of loci have employed only the Bayesian multi-species coalescent approach utilised by *BEAST (e.g. Barlow et al., 2013; Blanco-Pastor et al., 2012; Maddock et al., 2014a; Rocha et al., 2013; Satler et al., 2011; Wiens et al., 2012; Wood et al., 2011). However, here results of *BEAST analyses using different datasets were not in agreement with each other. It has been observed that a single locus can have a substantial impact on topologies recovered by *BEAST (Jockusch *et al.* 2015), something that *BEAST is supposed to be robust against (Heled & Drummond 2010).

The difference observed between the results of the species tree methodological approaches of the Bayesian (*BEAST) and maximum likelihood (ASTRAL) methods, when using the same datasets is likely due in

part to the algorithms used in each program. ASTRAL requires gene trees to be firstly reconstructed, whereas *BEAST jointly constructs gene trees and species trees. Even when using the strict algorithm in ASTRAL it is possible that the number of loci used in the datasets analysed here is too low to accurately infer topologies because the software was developed to produce species trees from genomic datasets consisting of hundreds of loci (Mirarab *et al.* 2014). Where ANL are used, the short length of these sequences could negatively impact species-tree phylogenetic inference. However, these short sequences should not negatively impact reconstruction based on distance matrix approaches and therefore this method is considered likely more useful when dealing with such data.

More studies are required to understand each of these approaches, their biases and benefits, and the features of datasets that make them particularly (in)appropriate. In the meantime, I recommend that researchers do not restrict themselves to a single approach only.

6.4.2 Seychelles caecilian systematics

Regardless of analytical considerations, the data appear unable to fully resolve the relationships among the Seychelles caecilians, as has also been the case with other molecular studies (Chapter 5; Gower et al., 2011; Hedges et al., 1993; Loader et al., 2007; Pyron and Wiens, 2011; Pyron, 2014; San Mauro et al., 2014, 2012; Wilkinson et al., 2003, 2002; Zhang and Wake, 2009). In almost all analyses the sister relationship between *Grandionsia larvata* and *G. sechellensis* was recovered. This clade has been recovered in other studies (e.g. Gower et al., 2011; Hedges et al., 1993; Loader et al.,

2007; Wilkinson et al., 2003, 2002) including Chapter 5 of this thesis using complete mitogenomes, and it is here considered a robust hypothesis.

The monophyly of *Hypogeophis rostratus* + *G. alternans* is recovered in all nuclear data analyses. This relationship has only previously been recovered by Wilkinson et al. (2003) in analyses of mt data, though without majority support. Other studies have recovered either *H. rostratus* (Hedges *et al.* 1993) or *G. alternans* (Gower *et al.* 2011) as sister to all remaining core species (albeit not well supported) but never together. A *G. alternans, H. brevis* and *H. rostratus* clade has been recovered in several studies (Wilkinson *et al.* 2002, 2003; Loader *et al.* 2007) but with low support. In the concatenated parsimony and complete Bayesian locus dataset analyses carried out here there was strong support for the sister relationships between between *H. brevis* + the two undescribed dwarf species *H.* cf. *brevis* CR and *H.* cf. *brevis* Praslin, and this is also considered a robust hypothesis here.

In the case of Seychelles caecilians, the disagreement among analytical approaches (and with some inconsistently recovered clades receiving high support in individual analyses) suggests that this is a difficult phylogenetic problem. The island setting in which the Seychelles caecilians diversified would have experienced repeated episodes of connection and vicariance during global sea level fluctuations (Colonna *et al.* 1996; Rohling *et al.* 1998; Siddall *et al.* 2003; Camoin *et al.* 2004; Miller *et al.* 2005). This repeated dis- and reconnection could potentially have allowed species to interbreed at these reconnection events prior to reproductive isolation being achieved. This potential exchange of alleles among some populations during the early evolution of the radiation could have caused a mixed history in allelic

variation. This would have resulted in a hard polytomy (e.g. see Olave et al., 2015; Poe and Chubb, 2004; Purvis and Garland, 1993), which may be why inference of species-level relationships in this case is difficult. A real hard polytomy by means of three or more lineages diverging at a similar time period could be to blame for the pattern observed in the dataset presented here, and it could in fact be a true representation of the ancestry of the radiation i.e. multiple branches coming from one node of the phylogenetic tree. The potential for this divergence during a relatively narrow but overlapping timeframe could be the reason for the differing ecologies/life histories displayed in the group having evolved in relative isolation of each other prior to a vicariance event reconnecting them. The Seychelles caecilians are a relatively ancient radiation, further exacerbating the problem of phylogenetic resolution.

6.4.3 Taxonomy

The reclassification by Wilkinson et al. (2011) of *Grandisonia* (*Hypogeophis*) *brevis* to the genus *Hypogeophis* was based on few morphological features rather than compelling phylogenetic evidence. Because of the findings herein that the type species of *Hypogeophis* Peters, 1880 (= *H. rostratus* (Cuvier, 1829)) and the type species of *Grandisonia* Taylor, 1968 (= *G. alternans* (Stejneger, 1893)) are recovered as sister taxa in all nuclear data analyses, I here relegate *Grandisonia* to the junior subjective synonymy of *Hypogeophis.* Wilkinson et al.'s (2011) morphological diagnosis for their revised concept of *Hypogeophis* (the only indotyphlids with the combination of eyes not covered by bone, tentacular groove covered by bone,

and mesethmoid not massively exposed between frontals) no longer holds for my new concept of the genus, such that a new morphological diagnosis will need to be established for *Hypogeophis*. The taxonomic solution proposed here tackles the probable non-monophyly of both *Hypogeophis* and *Grandisonia* as currently conceived, particularly in the light of ongoing difficulties in resolving the phylogenetic relationships among their constituent species.

6.4.4 Conclusions

It seems likely that to fully resolve the relationships among the Seychelles caecilians with molecular data a genomics approach will need to be undertaken. In this study the resolution of the core Seychelles caecilians has improved with analyses of a much larger dataset. The new areas of resolution are the sister relationship between *G. alternans* and *H. rostratus,* and recognition of a clade of three dwarf species (including *H. brevis*). Moderate support is also found for the hypothesis that the *G. alternans* + *H. rostratus* clade is sister to the remaining core (non-*Praslinia*) species. Given that improved resolution here is likely causally associated with an increase in data, it seems likely that even larger datasets may resolve the topology further. Perhaps a genomics approach such as exon-capture (e.g. Bi et al., 2012) would be appropriate. In addition, I recommend that morphological data are more extensively pursued for phylogenetic evidence.

CHAPTER 7

General Discussion and Conclusions

Before starting the research presented in this thesis a general picture was emerging of intraspecific variation in Seychelles organisms that was dominated by inter-island variation influenced mostly by the width and depth of intermittent marine barriers. This was especially clearly seen in studies of multiple lineages of lizard (Radtkey 1996; Rocha *et al.* 2010a, 2011, 2013a; Valente *et al.* 2014), in which variation is partitioned largely into northern and southern island groups. Populations on the southwestern island of Silhouette and eastern island of Fregate were also shown to be somewhat distinct, in association with their more peripheral geographical location and deeper marine barriers. Given that amphibians are expected, in general, to be less vagile than reptiles (especially in a marine island setting), it might have been predicted that variation in Seychelles amphibians would be even more strongly partitioned geographically – with the same island groups as discovered for lizards but perhaps with spatial structuring also within the northern and southern island groups. Preliminary studies of the sooglossid frogs (Van Der Meijden *et al.* 2007; Taylor *et al.* 2012; J. Labisko unpublished data) seem to match this expectation, but there were little or no data for the other native amphibians, the treefrog and caecilians. Thus, the major questions that this thesis aimed to answer were: what are the patterns of morphological and genetic variation in Seychelles amphibians? What can these patterns tell us about the diversification of these groups? Can generalities be drawn about diversification of Seychelles organisms? What do commonalities and

exceptions in these patterns mean for our understanding of evolution more broadly?

7.1 Achievements and limitations

At the outset of this research, little information was available on the variation and diversification of the Seychelles treefrog *Tachycnemis* and the radiation of caecilians of the genera *Praslinia, Hypogeophis* and *Grandisonia.* The treefrog was known to occur on four islands and to display substantial morphological variation that is strongly geographically partitioned (Nussbaum & Wu 1995). Nothing was known about genetic variation, and this hampered efforts to understand the causes and taxonomic implications of the morphological data. The phylogenetic relationships and biogeographic origins of the treefrog had been tested only in a limited fashion. The Seychelles caecilians were known to comprise a radiation with Gondwana origins (Nussbaum 1984a). The six nominal species had contrasting but overlapping distributions and understanding of within- and among-island variation was little studied and very poorly known. The phylogenetic relationships among the species were very incompletely resolved (Gower et al., 2011; Loader et al., 2007; San Mauro et al., 2012, 2014; Wilkinson et al., 2002, 2003; Zhang & Wake, 2009).

Research presented in this thesis has discovered, documented and interpreted variation in the treefrog *Tachycnemis* and the radiation of caecilians in the genera *Praslinia, Hypogeophis* and *Grandisonia.* The aims set out in Section 1.6 of this thesis have mostly been achieved. Data on morphological (Chapter 3) and molecular (Chapters 2 and 4) intraspecific variation have been extensively generated and analysed, and major patterns

documented. These data and analyses have allowed current taxonomies and classifications to be tested, and two new species were discovered. Similarities and differences among geographical patterns of pheno- and genotypic variation have been discovered. As a result, knowledge of the Seychelles amphibian fauna has advanced greatly. The thesis met two substantial challenges to meeting its aims. Firstly, there were notable discrepancies between some morphological and mitochondrial and nuclear intraspecific data sets, and among patterns of geographic intraspecific variation for different species – and this complicated the inference of evolutionary processes to explain the patterns, raising many further questions. Second, understanding the diversification of the caecilians was limited by the inability to determine a compelling resolution of their phylogenetic relationships, despite considerable effort.

Nevertheless, some progress was made in the molecular phylogenetics of Seychelles caecilians. Evidence was found for the discovery of two undescribed dwarf species (*H.* cf*. brevis* Praslin and *H.* cf*. brevis* CR) and these comprise a clade with the nominal dwarf species *H. brevis* with the two Mahé dwarves being sister species (Chapter 6). The previously inferred sister relationships between *G. larvata* and *G. sechellensis,* and between *Praslinia cooperi* and all other Seychelles species both receive further support (Chapters 5 and 6). Strong novel evidence is reported found for a sister relationship between *G. alternans* and *H. rostratus* (Chapter 6). Thus, the remaining lack of resolution is in the relationships among the dwarf, *larvata* + *sechellensis* and *rostratus* + *alternans* clades. A possible explanation for the difficulty in resolving relationships among Seychelles caecilian species is that

some of the divergences are genuinely hard polytomies (e.g. see Olave et al., 2015; Poe and Chubb, 2004; Purvis and Garland, 1993) caused by parallel diversification . If so, it might be impossible to resolve the phylogeny, even with additional data, though even if large amounts of gene flow occurred during speciation, increasing the number of loci may make it possible to resolve relationships among the non-*Praslinia* Seychelles caecilians.

7.2 Patterns of diversity and diversification among different Seychelles taxa

The data and analytical results presented in this thesis identify varying patterns of diversity and diversification among the Seychelles biota. The results in this thesis reject the hypothesis that there is a common pattern of intraspecific variation among ecologically similar, co-distributed species – contrary to findings for the Seychelles gecko and skink lizards (Radtkey 1996; Rocha *et al.* 2010a, 2011, 2013b; Valente *et al.* 2014).

Considering the findings for reptiles that patterns of intraspecific geographic variation in different groups (lizards, snakes and testudines) vary but are similar among species that have similar ecologies, it is unsurprising that the frogs and caecilians have different patterns of geographic structure. However, the hypothesis that within the frogs and caecilians, species will share similar patterns of geographic variation does not hold. Patterns of intraspecific geographic variation in the sooglossid frogs (Taylor *et al.* 2012; J. Labisko unpublished data) and the Seychelles treeefrog (Chapter 2) are notably dissimilar. All species of sooglossid frog that are co-distributed across multiple islands show high levels of genetic distinctiveness on each island

population (J. Labisko unpublished data). In contrast the treefrog, although showing molecular variation, does not show evidence of island-level geographic structure in genetic data. It should be noted that the ecologies of the sooglossids and treefrog are disparate, with sooglossids being direct developers restricted to leaf-litter in high elevation forest (Nussbaum 1984a), whereas the treefrog is more arboreal, and has aquatic larvae, so that populations are restricted to close proximity to permanent water bodies from sea level up to higher elevations (Nussbaum & Wu 1995). Being able to tolerate low elevations and having aquatic larvae might have facilitated greater admixture among populations of *T. seychellensis* on separate islands when low sea levels connected these landmasses.

The Seychelles caecilian species also display notably different patterns of intraspecific variation despite having broadly similar ecologies (fossorial, carnivorous, preferring moist forest habitats and many distributions overlapping). The conflicting patterns of diversification among Seychelles organisms are seemingly caused by different events during different time periods. The influence of these different events is evident in the most comparable of any Seychelles dataset, the caecilians (Chapters 3 and 4). Relative dating of molecular data in the caecilians (Chapter 4) demonstrates that even deep basal genetic splits within species (e.g. 7.6% mitochondrial sequence divergence in *G. alternans* and 5% in *G. larvata*) did not occur during the same period in time. Though it cannot be ruled out that some island populations dispersed and shared alleles with conspecifics in some species (e.g. *Praslinia cooperi*) during low stands in sea level as little as 10 Ka (Colonna et al. 1996; Rohling et al. 1998; Siddall et al. 2003; Camoin et al.

2004; Miller et al. 2005) it seems highly unlikely, due to the deep genetic splits, that all island population of each species would have.

When intraspecific morphological and molecular data are compared for the treefrog (Chapter 2) and caecilians (Chapters 3 and 4), particularly in the species with the largest genetic divergences (*G. alternans* and *G. larvata*), conflicting signals of variation are observed. The conflict suggests that morphological divergence may be a reflection of ecological adaptation overprinting signal of lineage divergence. Morphological vs. genetic disparity has not been reported for other Seychelles organisms but it supports the notion that to fully understand patterns and causes of intraspecific variation, multiple lines of evidence should be used.

7.3 Evolution and islands

What general conclusions can be drawn about the evolution of the Seychelles treefrog and caecilians based on this study and data published for other organisms? First, the profound cycle of sea-level rise and fall during the Pleistocene (Colonna et al. 1996; Rohling et al. 1998; Siddall et al. 2003; Camoin et al. 2004; Miller et al. 2005) seems to have had little impact in terms of driving lineage diversification. We have very little insight into how extinction might have masked events, but there does not seem to be much evidence, based on diversification levels, supporting speciation within any treefrog or caecilian within the Seychelles in the recent past. Second, although there are notable variations among patterns of within-lineage variation and diversification these can be explained post hoc by reasonable hypotheses. For example, geographic breaks in intraspecific variation (e.g., between Frégate

and elsewhere; between Silhouette and elsewhere; between northern and central Mahé) are aligned with more topographically profound marine or terrestrial barriers, and species with seemingly the greatest amount of inter-island gene flow (*T. seychellensis* and *P. cooperi*) are the only Seychelles amphibians with fully aquatic larvae. The main surprise is therefore not in the individual patterns but that not all species were affected the same way when facing the same abiotic changes. Thus, overall the system does not appear to be broadly predictable, with individual species having distinctive histories.

Other islands and island-like systems have been viewed as microcosms from which broader evolutionary generalities can be extrapolated (e.g. Lamichhaney *et al.* 2015). This is one of the main attractions of studying island evolution (see Chapter 1). This is perhaps challenged by the notable stochastic evolution of the Seychelles treefrog and caecilians discovered here. However, it is not yet clear the extent to which this is peculiar to these amphibians on these islands. The presence of relatively ancient amphibian radiations and of caecilians on islands is highly unusual and more detailed comparative studies of these and other taxa and required.

7.4 Taxonomy of the Seychelles treefrog and Seychelles caecilians

The results of this thesis have some profound implications for the taxonomy of the Seychelles treefrog and Seychelles caecilians, including the recognition of new species, the synonymising of a genus and the identity of potentially nominal subspecies. Evidence for the recognition of a single species of Seychelles treefrog is compelling (Chapter 2) despite the previously discovered substantial morphological differences among populations

(Nussbaum & Wu 1995). The results of Chapter 3 are also consistent with the recognition of the monotypic genus *Tachycnemis* because *T. seychellensis* (Seychelles treefrog) is recovered as most closely related to but lying outside a monophyletic *Heterixalus*.

Within the Seychelles caecilians the most notable taxonomic result is the discovery of two new dwarf species similar in morphology (Chapter 3) and genetics (Chapters 4 and 6) to *Hypogeophis brevis*. The three dwarf species are allopatric, though one of the new species, *H.* cf. *brevis* CR, occurs very close to populations of *H. brevis sensu stricto* on the southern island of Mahé. The second undescribed dwarf species, *H.* cf. *brevis* Praslin, occurs on the eastern half of the northern island Praslin at lower elevations than either *H. brevis sensu stricto* and or *H.* cf. *brevis* CR. These two new species are being formally described elsewhere.

It has become relatively common practice within amphibian phylogenetics to consider candidate species as those showing 3% sequence divergence (or less in certain cases) compared to in other taxonomic groups that consider a 6% threshold in order to be considered as a candidate species (see Fouquet et al., 2007; Funk et al., 2012). It is my opinion that there should be clear evidence from multiple independent sources of data (where available) to consider a new species to be valid e.g. multiple loci, morphology and ecology. Though deep intraspecific genetic splits were observed in *Grandisonia alternans*, *G. larvata* and *H. rostratus* (Chapter 4) it is my opinion that these units should not be split into further species-level taxa at this time. In *G. alternans* and *G. larvata* the deep basal genetic split is not matched by morphological differentiation, especially when only morphometric data are

considered (Chapter 3) but it cannot be ruled out that these are cryptic species. This distinction between the deep genetic lineages is also confounded by the possibility of a hybridisation zone in *G. alternans* between the two mitochondrial lineages on the southern versant of the Morne Seychellois National Park on Mahé. However, hybridisation has been reported among sympatric species in other radiations (e.g. Lamichhaney *et al.* 2015; Ford *et al.* 2015). However, these taxa could be considered to be unconfirmed candidate species pending further work.

Based on overlapping molecular and morphological results three distinct lineages can be recognised within *H. rostratus* (Chapters 3 and 4). Each of these lineages has been identified previously (based on meristic morphological data) and described as subspecies. Thus, *H. r. praslini* could be applied for the northern lineage (Parker 1958) consisting of populations on the islands of Praslin, La Digue, Curieuse, Félicité, Grand Soeur and Petite Soeur; *H. r. rostratus* for the southern lineage (Parker 1958) occurring on the islands of Mahé, Silhouette, Cerf and Saint Anne; and perhaps *H. r. guentheri* (Boulenger 1882) for specimens from the easternmost island of Frégate. The exact locality of the holotype of *H. r. guentheri* is not known (its original description was mistakenly reported to be from Zanzibar), but Parker (1958) suggested that it originated instead from the island of Frégate based solely on vertebral number in comparison to other specimens from known Seychelles localities. The holotype of the other nominal subspecies *H. r. lionneti* is a morphologically disparate individual (Taylor 1969), supposedly from Mahé. The type specimens of the nominal subspecies of *H. rostratus* were not able to be included in the molecular analyses reported here, and they were also not

included in morphological analyses. Thus, additional research is probably required before the various subspecies names could be applied with full confidence, putting aside whether it is useful to formally name subspecific taxa.

As discussed in Chapter 6, based on the non-monophyly of both *Hypogeophis* and *Grandisonia* (Chapters 4, 5 and 6) it is recommended that *Grandisonia* is considered a junior synonym of *Hypogeophis*. Both *H. rostratus* and *G. alternans* are the type species of their respective genera (Peters 1880; Taylor 1968) but are recovered robustly as sister species (Chapter 6). Throughout the rest of this chapter *Hypogeophis* will be used for all Seychelles caecilians except *Praslinia cooperi*, which is supported as being the sister taxon to all other Seychelles caecilians in this thesis and elsewhere (Gower et al., 2011; Loader et al., 2007; San Mauro et al., 2012, 2014; Wilkinson et al., 2002, 2003; Zhang & Wake, 2009).

7.5 Implications for the conservation biology of Seychelles amphibians?

The results of this thesis demonstrate the importance of studies of intraspecific variation in order to assess the distribution and potential fate of lineages and to identify priority areas of concern. Notably, species or populations that are restricted to small geographic areas (such as single islands or populations) are most vulnerable to extinction. Among the species studied in this thesis, only *H. brevis* and *P. cooperi* are considered endangered on the IUCN Red List; all other species are currently registered as Least Concern, and those categorisations rest primarily on geographic range size. Within the taxa studied here, several species and intraspecific lineages seemingly meet the

criterion of very small, isolated ranges and should be considered as evolutionary significant units (ESU) for conservation purposes. Those species and lineages that have seemingly small ranges and that are considered as being potentially threatened include: all of the species of the *H. brevis* group, *H. alternans* and *H. larvata* on the island of Silhouette, *H. alternans* from northern Mahé, *H. rostratus* on the island of Frégate, and the three island populations of *H. sechellensis* on Mahé, Silhouette and Praslin. Although *P. cooperi* is distributed across two islands its specialist ecology (restricted to higher elevation forests near watercourses) and seemingly low abundance (pers. obs.) also makes it vulnerable.

Among the threats to the Seychelles amphibian fauna is the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), which has decimated many amphibian populations globally (Berger *et al.* 1998; Skerratt *et al.* 2007). Only recently has *Bd* been documented in caecilians (Gower *et al.* 2013), something likely attributed to a lack of dedicated studies in the group. Although *Bd* has not been detected within the Seychelles (Labisko *et al.* 2015: including anaysis of samples collected during this study), the amount of international travel to the Seychelles (Quanz *et al.* 2009) from *Bd* positive countries means that it is likely only a matter of time before it arrives unless strict preventative measures are put in place. The susceptibility of the Seychelles amphibian fauna to lethal chytridiomycosis is unknown.

Habitat loss, climate change and invasive species all likely threaten the amphibian fauna of the Seychelles. The historical destruction of the native habitat on the granitic Seychelles is well documented, with complete destruction occurring on some of the islands (Baker 1877; Vesey-Fitzgerald

1940; Swabey 1970; Chang Seng & Guillande 2008; Kueffer *et al.* 2013). Despite the small amount of native habitat remaining on the Seychelles, the extant amphibian species seem to be able to cope with this destruction, as long as some forested or forest-like habitat remains – evident by the fact that much of Mahé was completely deforested by the early 19th century (Kueffer *et al.* 2013) but that many species are still present. Some species are even able to thrive in human modified habitats that are not forested, including the caecilians *H. alternans, H. larvata*, and especially *H. rostratus* (Nussbaum 1984; pers. obs.).

Besides invasive plants (Kueffer *et al.* 2013), introduced animals such as rats, tenrecs and crazy ants (Kaiser-Bunbury *et al.* 2014) likely pose some of the greatest threats to the Seychelles amphibians. It is unknown the direct impact that tenrecs and rats have on amphibian numbers but on the island of Frégate, where tenrecs and rats are absent, caecilian densities are noticeably higher (pers. obs.). The island of Silhouette, though containing rats has no records of tenrecs, and the densities of caecilians encountered here are also comparatively high (pers. obs.).

Climate change is a big threat to island amphibians. Species with restricted distributions are particularly vulnerable to the effects of climate change, especially those that are restricted to higher elevations (Pounds *et al.* 1999). The most threatened amphibian studied in this thesis in this respect is probably *H.* cf. *brevis* CR, which appears to be restricted to the high peak of Congo Rouge on Mahé. The only habitat that is similar to Congo Rouge is the highest reaches of the adjacent and higher Morne Seychellois though this has yet to be surveyed and *H.* cf. *brevis* CR is yet to be confirmed to occur there.

Increased temperatures have forced mountain dwelling species in other areas of the world to shift to higher elevations (e.g. Pounds *et al.* 1999; Klanderud & Birks 2003; Lenoir *et al.* 2008). Climate change could therefore push *H.* cf. *brevis* CR to extinction because there is very little room for an altitudinal range increase.

In order to preserve the biodiversity of the Seychelles amphibians detailed and achievable management plans need to be put in place. This could include both in- and ex situ conservation measures for each of the ESUs identified from the molecular and morphological data, especially those that are most at risk. The data presented in both morphology and molecules now allow for conservation biologists to decide on adequate management plans. Management strategies can now be designed around conserving finer-scale intraspecific variation. The data presented also has implications for the translocation of animals should near-extinction occur between populations on islands.

7.6 Future directions

This thesis provides substantial advances in knowledge of the evolution of Seychelles amphibians, but there is a lot of work to be done before a thorough understanding will be achieved. In particular, I propose that further research is now needed on details of distribution, particulars of intraspecific variation, ecology and population genetics for at least some species.

It is possible that we still do not fully know the ranges of all of the species and whether additional species remain undiscovered (except for the treefrog). This is especially the case for caecilians and sooglossid frogs which

are cryptic and hard to observe in nature. This is evident by the recent discoveries of two new sooglossid frogs (Gerlach & Willi 2002; Taylor *et al.* 2012) and the two dwarf caecilians (this thesis). During the fieldwork for this thesis a new island record for *H. alternans* on Praslin was also found. Additional taxon-specific surveys are encouraged, especially in remote and/or scarcely sampled areas such as valleys and high peaks in Morne Seychellois National Park, Mahé. Although the granitic Seychelles do not constitute a large landmass, several areas not adjacent to roads are surprisingly difficult to access. Few caecilian surveys have also been carried out on the smaller, drier islands–these are often expensive and/or difficult to visit.

Better knowledge of the ecological diversity of Seychelles caecilians would better allow key questions in their evolution to be addressed. Ecological questions of priority include: do diets and seasonality vary among species/islands/sexes? Do life history modes predict intraspecific diversity and diversification? Is there character displacement in sympatry? What is the functional (ecological) significance of those morphological characters that have been found to vary within species among islands?

Within the caecilians, more detailed studies are required of species showing high levels of intraspecific geographic divergences (e.g. *H. alternans* and *H. larvata*). These studies would help to try and complete phylogenetic resolution, clarify evolutionary patterns within species, and further stabilise taxonomy. Denser geographic sampling is required in some cases, and generating data for additional phenotypic (e.g., caecilian osteology; treefrog vocalisations) and molecular (additional loci) characters would be worthwhile.

Detailed population genetics analyses would facilitate fuller understanding of morphological and molecular incongruence and the extent to which immigration and dispersal are occurring among populations within and among islands. The work presented in this thesis has defined the main units and focused attention on cases where fine scale studies of population dynamics and gene flow would be most beneficial. Appropriate population genetics datasets will need to be generated, for example using microsatellites and/or genomic single nucleotide polymorphism (SNP) data.

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APPENDIX 1

Details of samples used in Chapter 2

GenBank accession numbers and locality information for *Tachycnemis seychellensis* and *Heterixalus spp*. Island localities given only for Seychelles specimens (all *Heterixalus* are from Madagascar). *Heterixalus* data from previous studies; *Tachycnemis* data newly generated. Specimens with RAN prefix are from the tissue collection of the University of Michigan Museum of Zoology; other specimens are as reported by Wollenberg et al. (2007).

Species	Island	Specimen	mtDNA (*16s*, *cytb, cox1*)	*rag1*	*pomc*	*tyr*	*rho*
T. seychellensis	Mahé	RAN30761	KJ551572, KJ551624, KJ551676	KJ551817	KJ551713	KJ551805	
T. seychellensis	Mahé	RAN30762	KJ551573, KJ551625, KJ551677	KJ551818	KJ551714	KJ551806	
T. seychellensis	Mahé	RAN30763	KJ551574, KJ551626, KJ551678	KJ551819	KJ551715	KJ551807	KJ551789
T. seychellensis	Mahé	RAN30764	KJ551575, KJ551627, KJ551679	KJ551820	KJ551716		KJ551793
T. seychellensis	Mahé	RAN30765	KJ551576, KJ551628, KJ551680	KJ551821	KJ551717		KJ551765
T. seychellensis	Mahé	RAN30766	KJ551571, KJ551623, KJ551675		KJ551718		KJ551788
T. seychellensis	Mahé	RAN30767	KJ551557, KJ551609, KJ551661		KJ551719		KJ551794
T. seychellensis	Mahé	RAN30769	KJ551558, KJ551610, KJ551662		KJ551720		KJ551795
T. seychellensis	Mahé	RAN30770	KJ551577, KJ551629, KJ551681		KJ551721		
T. seychellensis	Mahé	RAN30771	KJ551559, KJ551611, KJ551663		KJ551722		
T. seychellensis	Mahé	RAN30772	KJ551578, KJ551630, KJ551682		KJ551723		KJ551774
T. seychellensis	Mahé	RAN30773	KJ551579, KJ551631, KJ551683		KJ551724		KJ551791
T. seychellensis	Mahé	RAN30774	KJ551560, KJ551612, KJ551664		KJ551725		KJ551787
T. seychellensis	Mahé	RAN30775	KJ551561, KJ551613, KJ551665		KJ551726		
T. seychellensis	Mahé	RAN30776	KJ551580, KJ551632, KJ551684		KJ551727		KJ551786
T. seychellensis	Silhouette	RAN30789	KJ551581, KJ551633, KJ551685	KJ551822	KJ551728	KJ551808	
T. seychellensis	Silhouette	RAN30790	KJ551582, KJ551634, KJ551686	KJ551823	KJ551729	KJ551809	KJ551796
T. seychellensis	Silhouette	RAN30791	KJ551583, KJ551635, KJ551687	KJ551824	KJ551730	KJ551810	KJ551797
T. seychellensis	Silhouette	RAN30792	KJ551570, KJ551622, KJ551674	KJ551825	KJ551731		KJ551798
T. seychellensis	Silhouette	RAN30793	KJ551569, KJ551621, KJ551673	KJ551826	KJ551732		KJ551766
T. seychellensis	Silhouette	RAN30794	KJ551568, KJ551620, KJ551672		KJ551733		KJ551799
T. seychellensis	Silhouette	RAN30795	KJ551567, KJ551619, KJ551671		KJ551734		KJ551785
T. seychellensis	Silhouette	RAN30796	KJ551566, KJ551618, KJ551670		KJ551735		KJ551800
T. seychellensis	Silhouette	RAN30797	KJ551584, KJ551636, KJ551688		KJ551736		KJ551801

T. seychellensis	Silhouette	RAN30798	KJ551585, KJ551637, KJ551689		KJ551737		KJ551802
T. seychellensis	Silhouette	RAN30799	KJ551586, KJ551638, KJ551690		KJ551738		
T. seychellensis	Silhouette	RAN30800	KJ551587, KJ551639, KJ551691		KJ551739		
T. seychellensis	Silhouette	RAN30801	KJ551588, KJ551640, KJ551692		KJ551740		KJ551767
T. seychellensis	Silhouette	RAN30802	KJ551589, KJ551641, KJ551693		KJ551741		KJ551768
T. seychellensis	Silhouette	RAN30803	KJ551590, KJ551642, KJ551694		KJ551742		KJ551792
T. seychellensis	Praslin	RAN30829	KJ551565, KJ551617, KJ551669		KJ551743	KJ551811	KJ551769
T. seychellensis	Praslin	RAN30830	KJ551598, KJ551650, KJ551702	KJ551827	KJ551744	KJ551812	KJ551770
T. seychellensis	Praslin	RAN30831	KJ551599, KJ551651, KJ551703	KJ551828	KJ551745	KJ551813	KJ551784
T. seychellensis	Praslin	RAN30832	KJ551600, KJ551652, KJ551704	KJ551829	KJ551746		KJ551803
T. seychellensis	Praslin	RAN30833	KJ551601, KJ551653, KJ551705	KJ551830	KJ551747		KJ551783
T. seychellensis	Praslin	RAN30834	KJ551602, KJ551654, KJ551706	KJ551831	KJ551748		KJ551782
T. seychellensis	Praslin	RAN30835	KJ551603, KJ551655, KJ551707		KJ551749		KJ551771
T. seychellensis	Praslin	RAN30836	KJ551604, KJ551656, KJ551708		KJ551750		KJ551781
T. seychellensis	Praslin	RAN30837	KJ551591, KJ551643, KJ551695		KJ551751		KJ551772
T. seychellensis	Praslin	RAN30838	KJ551605, KJ551657, KJ551709		KJ551752		
T. seychellensis	Praslin	RAN30839	KJ551564, KJ551616, KJ551668		KJ551753		KJ551790
T. seychellensis	Praslin	RAN30840	KJ551606, KJ551658, KJ551710		KJ551754		
T. seychellensis	Praslin	RAN30841	KJ551607, KJ551659, KJ551711		KJ551755		
T. seychellensis	Praslin	RAN30842	KJ551608, KJ551660, KJ551712		KJ551756		KJ551804
T. seychellensis	Praslin	RAN30843	KJ551592, KJ551644, KJ551696		KJ551757		KJ551773
T. seychellensis	La Digue	RAN30891	KJ551593, KJ551645, KJ551697	KJ551832	KJ551758	KJ551814	KJ551780
T. seychellensis	La Digue	RAN31659	KJ551563, KJ551615, KJ551667	KJ551833	KJ551759	KJ551815	KJ551779
T. seychellensis	La Digue	RAN31768	KJ551594, KJ551646, KJ551698	KJ551834	KJ551760	KJ551816	KJ551778
T. seychellensis	La Digue	RAN31857	KJ551595, KJ551647, KJ551699	KJ551835	KJ551761		KJ551777
T. seychellensis	La Digue	RAN31858	KJ551596, KJ551648, KJ551700	KJ551836	KJ551762		KJ551775
T. seychellensis	La Digue	RAN31859	KJ551597, KJ551649, KJ551701		KJ551763		KJ551776
T. seychellensis	La Digue	RAN31860	KJ551562, KJ551614, KJ551666		KJ551764		
Heterixalus andrakata		ZSM 508/2000	EF646676, EF646609, -	EF646559		EF646491	EF646491
H. andrakata		FGMV 2000.372	EF646680, EF646613, -	EF646563		EF646495	EF646530
H. andrakata		ZSM 566/2000	EF646677, EF646610, -	EF646560		EF646492	EF646527
H. betsileo		ZSM 356/2000	EF646672, EF646605, -	EF646555		EF646487	EF646522
H. betsileo		ZSM 682/2001	EF646661, EF646594, -	EF646545		EF646476	EF646511
H. betsileo		ZSM 718/2001	EF646668, EF646601, -	EF646551		EF646483	EF646518
H. betsileo		FGMV 2000.14	EF646671, EF646604, -	EF646554		EF646486	EF646521
H. luteostriatus		ZSM 697/2001	EF646666, EF646599, -	EF646549		EF646481	EF646516

H. luteostriatus	FGMV 2000.274	EF646665, EF646598, -	EF646548	EF646480	EF646515
H. luteostriatus	ZSM 426/2000	EF646685, EF646618, -	EF646568	EF646500	EF646535
H. madagascariensis	ZSM 569/2000	EF646678, EF646611, -	EF646561	EF646493	EF646528
H. madagascariensis	ZSM 568/2000	EF646682, EF646615, -	EF646565	EF646497	EF646532
H. madagascariensis	ZSM 684/2001	EF646659, EF646592, -	EF646543	EF646474	EF646509
H. madagascariensis	FGMV 2001.222	EF646660, EF646593, -	EF646544	EF646475	EF646510
H. punctatus	ZSM 683/2001	EF646670, EF646603, -	EF646553	EF646485	EF646520
H. punctatus	ZSM 349/2000	EF646662, EF646595, -	EF646546	EF646477	EF646512
H. punctatus	ZSM 571/2000	EF646679, EF646612, -	EF646562	EF646494	EF646529
H. punctatus	FGMV 2000.374	EF646681, EF646614, -	EF646564	EF646496	EF646531
H. punctatus	ZSM 572/2000	EF646683, EF646616, -	EF646566	EF646499	EF646533
H. rutenbergi	ZSM 361/2000	EF646673, EF646606, -	EF646556	EF646488	EF646523
H. rutenbergi	ZSM 789/2001	EF646667, EF646600, -	EF646550	EF646482	
H. tricolor	ZSM 700/2001	EF646664, EF646597, -	EF646547	EF646479	EF646514
H. tricolor	ZSM 463/2000	EF646674, EF646607, -	EF646557	EF646489	EF646524
H. tricolor	FGMV 2000.235	EF646675, EF646608, -	EF646558	EF646490	EF646525
H. variabilis	ZSM 425/2000	EF646687, EF646620, -	EF646570	EF646502	EF646537
H. variabilis	ZSM 608/2001	EF646669, EF646602, -	EF646552	EF646484	EF646519
H. variabilis	FGMV 2000.188	EF646688, EF646621, -	EF646571	EF646503	EF646538
H. variabilis	FGMV 2000.185	EF646686, EF646619, -	EF646569	EF646501	EF646536

APPENDIX 2

Published paper based on chapter 2: Maddock *et al.* (2013)

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Evolutionary origins and genetic variation of the Seychelles treefrog, *Tachycnemis seychellensis* (Duméril and Bibron, 1841) (Amphibia: Anura: Hyperoliidae)

Simon T. Maddock [a,b,*], Julia J. Day [a], Ronald A. Nussbaum [c], Mark Wilkinson [a,b], David J. Gower [b]

[a] Department of Genetics, Evolution and Environment, University College London, London WC1E 6BT, UK
[b] Department of Life Sciences, The Natural History Museum, London SW7 5BD, UK
[c] Museum of Zoology and Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109-1079, USA

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A B S T R A C T

The hyperoliid frog *Tachycnemis seychellensis*, the only species of its genus, is endemic to the four largest granitic islands of the Seychelles archipelago and is reliant on freshwater bodies for reproduction. Its presence in the Seychelles is thought to be the product of a transoceanic dispersal, diverging from the genus *Heterixalus*, its closest living relative (currently endemic to Madagascar), between approximately 10–35 Ma. A previous study documented substantial intraspecific morphological variation among island populations and also among populations within the largest island (Mahé). To assess intraspecific genetic variation and to infer the closest living relative(s) of *T. seychellensis*, DNA sequence data were generated for three mitochondrial and four nuclear markers. These data support a sister-group relationship between *T. seychellensis* and *Heterixalus*, with the divergence between the two occurring between approximately 11–19 Ma based on *cytb* *p*-distances. Low levels of genetic variation were found among major mitochondrial haplotype clades of *T. seychellensis* (maximum 0.7% *p*-distance concatenated mtDNA), and samples from each of the islands (except La Digue) comprised multiple mitochondrial haplotype clades. Two nuclear genes (*rag1* and *tyr*) showed no variation, and the other two (*rho* and *pomc*) lacked any notable geographic structuring, counter to patterns observed within presumably more vagile Seychelles taxa such as lizards. The low levels of genetic variation and phylogeographic structure support an interpretation that there is a single but morphologically highly variable species of Seychelles treefrog. The contrasting genetic and morphological intraspecific variation may be attributable to relatively recent admixture during low sea-level stands, ecophenotypic plasticity, local adaptation to different environmental conditions, and/or current and previously small population sizes. Low genetic phylogeographic structure but substantial morphological variation is unusual within anurans.

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1. Introduction

Due to their isolation from potential confounding factors, remote islands have long been considered to provide important arenas for investigating evolution (Darwin, 1859). Most evolutionary studies of island biotas have focused on geologically recent volcanic island groups that have never been in contact with a large, ancient mainland, for example the Galápagos and Hawaii (e.g. Darwin, 1859; Gillespie, 2002). In contrast, the Seychelles archipelago (1600 km east of the nearest continental landmass) is formed of both granitic and coralline islands. The current granitic Seychelles are the remaining emergent part of a continental fragment, previously part of Gondwana, that was associated with India and Madagascar when they separated from Africa during the Cretaceous. At least some of the granitic Seychelles have always had some emergent land since the breakup of the Gondwanan supercontinent. Much of the continental Seychelles is currently submerged at an average depth of 55 m below sea level, forming the microcontinent 'Seychellea', comprising a total area of 129,650 km^2 (Davies and Francis, 1964). During times of lowest stands in sea level (see Miller et al., 2005) all of the currently emergent granitic Seychelles would have been in contact. Fluctuations in sea level likely caused many episodes of dis- and reconnection among Seychelles islands, the most recent of which were within the last 10 ka (Colonna et al., 1996; Rohling et al., 1998; Siddall et al., 2003; Camoin et al., 2004; Miller et al., 2005). These

* Corresponding author at: Department of Life Sciences, The Natural History Museum, London SW7 5BD, UK.
E-mail address: s.t.maddock@gmail.com (S.T. Maddock).

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fluctuations can be expected to have had a substantial impact on the amounts and spatial patterns of genetic variation of the resident biota.

It is unsurprising that most remote islands lack an endemic amphibian fauna given that the osmotic properties of amphibian skin (e.g. Balinsky, 1981; Duellman and Trueb, 1986) likely reduce their dispersal capabilities over saltwater substantially (Bossuyt and Milinkovitch, 2001; Darwin, 1859; Myers, 1953; Nussbaum, 1984). The Seychelles, however, has an amphibian fauna (~12 species) comprising both frogs (Anura) and caecilians (Gymnophiona) that are restricted solely to the granitic islands (Nussbaum, 1984). Except for the widespread frog *Ptychadena mascareniensis* (Duméril and Bibron, 1841; Vences et al., 2004), all Seychelles amphibians are endemic. The endemic Seychelles frogs are confined to the four largest granitic islands of Mahé, Silhouette, Praslin and La Digue (Nussbaum and Wu, 1995, 2007; Taylor et al., 2012). Except for a preliminary study of sooglossid frogs (Taylor et al., 2012) molecular analyses have not yet been conducted to determine patterns of genetic variation among and within populations on different islands. Molecular techniques have been applied to several other Seychelles organisms, and substantial spatial structuring and deep genetic splits have been revealed, indicating the presence of cryptic lineages within several currently recognised lizard species (Rocha et al., 2010a,b, 2011, 2013; Valente et al., 2014), and a freshwater crab (Daniels, 2011).

Hyperoliidae is a pan-African family comprising >200 species in 17 or 18 genera (AmphibiaWeb, 2014; Frost, 2014) of small-medium sized treefrogs with representative species also found in the Seychelles and Madagascar (Vences et al., 2003a,b). The endemic Seychelles treefrog *Tachycnemis seychellensis*, the only species of its genus, is a sexually dimorphic, hyperoliid frog found on all four of the granitic islands of the Seychelles that support populations of frogs (Nussbaum and Wu, 1995). Like all hyperoliids *T. seychellensis* is an oviparous species with an aquatic larval stage, and it is restricted to areas close to water bodies (Nussbaum, 1984). The abundance and type of *T. seychellensis* habitat varies considerably across its range (Nussbaum and Wu, 1995). The southern islands of Mahé and Silhouette are higher (up to 905 and 750 m elevation, respectively), wetter and dominated by moist–wet forests, whereas the northern islands of Praslin (up to 367 m) and La Digue (333 m) are much lower and drier. Praslin has multiple rivers and streams, but La Digue lacks constant water sources at higher altitudes, and instead *T. seychellensis* is restricted here to marshy areas in the low-lying plateau on the west of this small island. The sizes of the four islands vary by more than an order of magnitude, ranging from 960 ha (La Digue) to 14,480 ha (Mahé), with Silhouette (1600 ha) and Praslin (4040 ha) somewhat intermediate.

Using univariate and multivariate analyses, Nussbaum and Wu (1995) discovered substantial external morphological variation among five populations of *T. seychellensis* from the four islands, including in adult body size and colouration, presence or absence of tubercles on various parts of the body and limbs, presence or absence of grooved digit discs, and several morphometric characters. Four morphometric characters, not dependant on sex, were found to vary significantly between all populations: internarial width, pes length, toe disc length, and length of metatarsal tubercle. An additional 10 male and two female characters varied significantly. Specimens from the more southerly islands of Mahé and Silhouette are morphologically the most similar to each other (Nussbaum and Wu, 1995). However, within Mahé (the only island for which more than one population was sampled), two populations of *T. seychellensis* (one marsh- and one stream-associated) only 1 km apart were as morphologically different from each other as they were to *T. seychellensis* on Silhouette. The populations of *T. seychellensis* on the more northerly islands (Praslin and La Digue) were morphometrically as distinct from each other as they were from the southern populations. Despite these large morphological differences, Nussbaum and Wu (1995) were impressed by (1) the fact that the four islands were likely connected as recently as 10 ka, (2) the intra-Mahé differences were as large as inter-island differences, (3) the substantial environmental differences across the four islands, and (4) the similar life history and bioacoustics of the different populations, and thus argued for the recognition of only a single species, one that has substantial and geographically structured morphological variation. Nussbaum & Wu's single-species hypothesis for *T. seychellensis* could be challenged by high genetic diversity and/or substantial phylogeographic structure.

Tachycnemis seychellensis has a complicated taxonomic history. Since Dubois (1981) the species has been included in the monotypic genus *Tachycnemis* Fitzinger, 1843 and the species name has been attributed to Duméril and Bibron (1841) with Tschudi's (1838) first use of the species name considered unavailable. Fitzinger (1843) established *Tachycnemis* only through bibliographic reference to its single included species (as described by Tschudi, 1838) without any explanation of his biological reasons (if any) for proposing the new genus. However, it has long been considered a phenotypically rather distinct hyperoliid (e.g. Günther, 1869), and Drewes (1984) hypothesised that it is the sister group of all other extant hyperoliids. More recently, based on analysis of concatenated mitochondrial DNA (mtDNA) and nuclear DNA (nuDNA), *T. seychellensis* has been inferred to be most closely related to the endemic Madagascan genus *Heterixalus* Laurent, 1944, which has 11 currently recognised species (Frost et al., 2006; Pyron and Wiens, 2011; Richards and Moore, 1996; Vences et al., 2003a,b; Wollenberg et al. 2007). However, although Wollenberg et al.'s (2007) main analysis of concatenated data recovered *Tachycnemis* and *Heterixalus* as sister taxa, five out of six trees inferred for the individual genes placed *Tachycnemis* within *Heterixalus*, although only *cox1* (mtDNA) and *rho* (nuDNA) did so with much support. Paraphyly of *Heterixalus* with respect to *Tachycnemis* was also found (though without strong support) by Vences et al. (2003b) in two mitochondrial gene trees. In contrast, these authors found that when three genes were concatenated, but using only two *Heterixalus* species, *Tachycnemis* and *Heterixalus* were sister taxa. Using multiple *Heterixalus* species in their analyses, Richards and Moore (1996), Frost et al. (2006), and Pyron and Wiens (2011) also recovered a *Tachycnemis–Heterixalus* sister-group relationship. Where trees for individual loci are discordant, coalescence-based methods can be expected to yield more accurate species phylogenies than multilocus concatenation (e.g. Edwards et al., 2007; Heled and Drummond, 2010; Kubatko and Degnan, 2007; Maddison and Knowles, 2006), but this latter approach has yet to be implemented in the case of *Tachycnemis* and *Heterixalus*.

Based on a sister-group relationship with *Heterixalus* and molecular dating analyses, the presence of *T. seychellensis* in the Seychelles is considered to originate from an overseas dispersal, with *Tachycnemis* diverging from its closest African/Madagascan relative an estimated 9.79–35.34 Ma (Crottini et al., 2012). This is in contrast to the sooglossid frogs that, as with the Seychelles caecilians, have probably been resident at least since Seychellea (the Seychelles microcontinent) was last part of Gondwana (Nussbaum, 1984).

Here we report phylogenetic analyses of mtDNA and nuDNA data (3228 base pairs (bp)) to (1) test the hypothesised sister-group relationship between *Heterixalus* and *Tachycnemis* and monophyly of the former genus, and (2) assess genetic variation within *T. seychellensis* across its range and test the hypothesis that it is a single, morphologically highly variable species.

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2. Methods

2.1. Taxon sampling

Tachycnemis seychellensis tissue samples (liver, heart and muscle, frozen and stored at −80 °C) were obtained from 52 voucher specimens from the Seychelles islands of Mahé (15 samples), Silhouette (15 samples), Praslin (15 samples) and La Digue (7 samples) between 1988 and 1991; these correspond to four of the five populations sampled by Nussbaum and Wu (1995) (tissues of only a single Mahé population from Mare aux Cochons were available). Vouchers and tissues are deposited in the University of Michigan Museum of Zoology, USA (UMMZ) (see Appendix for details).

2.2. Laboratory protocols

Genomic DNA was extracted from liver, heart and muscle samples from the 52 *T. seychellensis* samples using the Qiagen DNeasy™ Tissue Kit. Three mitochondrial gene fragments were sequenced for all samples: cytochrome *b* (*cytb*), cytochrome oxidase subunit 1 (*cox1*) and 16S rRNA (*16s*). Four nuclear loci were also sequenced: rhodopsin exon 1 (*rho*), recombination activating gene 1 (*rag1*), tyronsinase precursor (*tyr*) and pro-opiomelanocortin (*pomc*). The *rag1* and *tyr* sequences showed no variation within *T. seychellensis,* and thus only a subset of individuals from each locality were included in the analyses of the relationships between *Tachycnemis* and *Heterixalus*.

Primer information is given in Table 1. Sequences were amplified using the polymerase chain reaction (PCR) with a total reaction volume of 15 µl: 1.5 µl of Bioline Buffer, 0.75 µl of MgCl2, 0.15 µl of dNTPs, 0.15 µl of Taq, 06 µl of both the forward and reverse primers, 0.6 µl of template DNA, and 10.65 µl ddH_2O. Cycling conditions were: denature at 94 °C for 60s; followed by 35 (*16s*, *cytb*) or 40 (*cox1*, *tyr*, *pomc*, *rag1*) cycles of denaturing at 94 °C for 30 s, annealing at 48 °C (*cox1*), 50 °C (*16s*), 52 °C (*cytb*), 56 °C (*rag1*), 60 °C (*rho*), or 62 °C (*tyr*, *pomc*) for 30 s, and extending at 72 °C for 30 s; and a final extending step of 72 °C for 5 min.

Table 1
Primers used in this study for PCR and sequencing.

Gene fragment	Primer	Sequence (5′–3′)
16s	16SA-L [a]	CGCCTGTTTATCAAAAACAT
	16SB-H [a]	CCGGTCTGAACTCAGATCACGT
cox1	Amp-P3 F [b]	CAATACCAAACCCCCTTRTTYGTWTGATC
	Amp-P3 R [b]	GCTTCTCARATAATAAATATYAT
cytb	L14841 [c]	CTCCCAGCCCCATCCAACATCTCAGCATGATGAAACTTCG
	CB3H [d]	GGCAAATAGGAAGTATCATTCTG
pomc	POMC-1 [e]	GAATGTATYAAAGMMTGCAAGATGGWCCT
	POMC-2 [e]	TAYTGRCCCTTYTTGTGGGCRTT
tyr	Tyr1C [f]	GGCAGAGGAWCRTGCCAAGATGT
	Tyr1G [f]	TGCTGGGCRTCTCTCCARTCCCA
rho	Rhod1A[f]	ACCATGAACGGAACAGAAGGYCC
	Rhod1D[f]	GTAGCGAAGAARCCTTCAAMGTA
rag1	Amp-RAG1 F [b]	AGCTGCAGYCARTACCAYAARATGTA
	Amp-RAG1 R1 [b]	AACTCAGCTGCATTKCCAATRTCACA

[a] Palumbi et al. (1991).
[b] San Mauro et al. (2004).
[c] Kocher et al. (1989).
[d] Moritz et al. (1992).
[e] Wiens et al. (2005).
[f] Bossuyt and Milinkovitch (2000).

2.3. Genetic variation within T. seychellensis

Sequences were proof-read using Sequencher v.4.8 and initially aligned using ClustalX v.2.0 (Larkin et al., 2007) using default settings before being checked by eye. All genes except the non-protein-coding *16s* were checked for pseudogenes and insertions by searching for stop codons and indels (e.g. Zhang and Hewitt, 1996) in MEGA5 (Tamura et al., 2011). The program DAMBE (Xia and Xie, 2001) was used to test for saturation using the test of Xia et al. (2003) across the different codon positions and the combined dataset.

To infer the phylogenetic relationships within *T. seychellensis* for the mitochondrial locus, we used Bayesian inference (BI) implemented in BEAST v.1.7.4 (Drummond et al., 2012). No outgroup taxa were used because BEAST estimates the position of the root in the tree assuming a molecular clock (Heled and Drummond, 2010). Input XML files were generated for BEAST analyses using BEAUti v.1.7.4. We selected best partitioning strategies and BEAST-compatible substitution models using PartionFinder (Lanfear et al., 2012).

The coalescent tree prior with exponential growth was used in BEAST based on the assumption that, after an initial colonisation, *T. seychellensis* likely expanded its range. Following the results of initial runs, uncorrelated relaxed clocks were rejected for all partitions and a strict clock implemented because constant rates could not be rejected. Two MCMC chains were run for 1 x 10^8 generations for each partitioning strategy, with trees sampled every 10,000 generations to ensure convergence; this gave a total of 10,000 output trees per run. Convergence was checked by manual observation of the trace plots and ESS scores using Tracer v.1.5 (Rambaut and Drummond, 2009). All BEAST analyses were performed using the CIPRES Science Gateway v.3.1 (Miller et al., 2010).

To infer allelic phases from polymorphic sites in the nuDNA, the program PHASE v.2.1 (Stephens et al., 2001; Stephens and Scheet, 2005) was used, with input files created using seqPHASE (Flot, 2010). Haplotype networks under the median-joining algorithm (Bandelt et al., 1999) were produced to display intraspecific variation for *T. seychellensis* for the *pomc* and *rho* loci using the program NETWORK v.4.611 (fluxus-engineering.com).

Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997) neutrality tests were used to investigate historical demographic properties in each island population of *T. seychellensis*. Negative values indicate a recent population expansion, values close to zero indicate a stable population, and any values considerably over zero indicate a recent population decrease. Both *D* and *Fs* were calculated using Arlequin v.3.5.1.3 (Excoffier and Lischer, 2010). Input files for Arlequin were created using PGDSpider v.2.0.3.0 (Lischer and Excoffier, 2012).

2.4. Testing monophyly of Heterixalus

The multispecies coalescent method as implemented in *BEAST (Heled and Drummond, 2010) was used to infer the species trees

Table 2
Partitioning schemes and substitution models for the *Tachycnemis seychellensis* intraspecific mtDNA dataset. Numbers in parentheses refer to codon position.

	Partition scheme	Substitution models
AIC/AICc	*16s*	TrN
	cytb (1), *cox1* (1)	HKY
	cytb (2), *cox1* (2)	HKY
	cytb (3)	TrN
	cox1 (3)	TrN + G
BIC	*16s*, *cytb* (1), *cox1* (1)	HKY
	cytb (2), *cox1* (2)	HKY
	cytb (3), *cox1* (3)	TrN + G

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Table 3
Best-fit substitution models for partitions for the multispecies coalescent analysis.

	Partition	Substitution models
Locus partitions	*mtDNA*	GTR + G
	rag1	TrN + I
	rho	GTR + G
	tyr	SYM + I + G

for *Tachycnemis* and *Heterixalus* spp., treating mtDNA (*cytb*, *cox1* and *16s*), *tyr*, *rag1* and *rho* as four separately evolving loci. Sequence data for *Heterixalus* spp. were previously published (Wollenberg et al., 2007) and obtained from GenBank, and those for *Tachycnemis* were newly generated. It is recommended to include a minimum of two specimens per species for *BEAST analyses so that there is a coalescent event with which to estimate population size (Heled and Drummond, 2010), but this was not possible for all species of *Heterixalus* because of inadequate specimen and/or character sampling in GenBank. For this reason *H. alboguttatus*, *H. boettgeri* and *H. carbonei* were excluded from these analyses. The remaining taxa nonetheless included representatives of all five *Heterixalus* species groups identified by Wollenberg et al. (2007).

Many studies using multilocus datasets do not partition by codon position, but we ran two sets of analyses in order to test for discrepancies between this *ad hoc* method and the optimal partitioning strategy identified by PartitionFinder (Table 3). Due to over parameterization, convergence was not reached in the identified optimal partitioning scheme in further analyses and therefore only the results of the locus partitioned analysis are used.

Preliminary analyses of the locus-partitioned dataset suggested that strict clocks be implemented for the mtDNA and *rhod* partitions and an uncorrelated lognormal relaxed clock for the *tyr* and *rag1*. Rates for molecular clocks were initially set at default (1.0) for all partitions and estimated relative to the mtDNA partition. Two MCMC chains were run for 2×10^8 generations, with trees sampled every 10,000 generations, to ensure convergence was reached the first 5% were discarded as burn-in, although convergence was reached prior to this cut-off. The species-tree Yule-process prior was used with the piecewise linear and constant-root population-size model. Convergence of all parameters was verified using Tracer v.1.5 (Rambaut and Drummond, 2009).

3. Results

3.1. Monophyly of Heterixalus?

We aligned 1500 bp of mtDNA (consisting of 424 variable sites (v.s.), of which 391 were parsimony informative (p.s.)), 760 bp of *rag1* (62 v.s., 48 p.s.), 357 bp of *rho* (30 v.s., 15 p.s.), and 611 bp of *tyr* (56 v.s., 45 p.s.) giving a total sequence length of 3228 bp. Partitioning schemes and nucleotide models used in analyses are presented in Table 3.

Partitioning the *BEAST dataset by linked loci provided evidence for *T. seychellensis* being the sister taxon to a monophyletic *Heterixalus*; a sister-group relationship between *H. madagascariensis* and *H. punctatus*; for *H. andrakata* being the sister taxon to *H. tricolor* + *H. variabilis*; and for *H. betsileo* being the sister taxon to the *andrakata–tricolor–variabilis* clade (Fig. 1). The relationships of the remaining two species (*H. rutenbergi* and *H. luteostriatus*) are unresolved (Fig. 1).

Among *Heterixalus* species and clades, mean *p*-distances for *cytb* range from 3% to 19.5%, with the *p*-distance between *Tachycnemis* and *Heterixalus* being 21% (Fig. 1). Given an approximate rate of 0.6–1% per million years for *cytb* in amphibians (see Elmer et al., 2007), this marker indicates that *T. seychellensis* diverged from its closest sampled relative in the region of 11.5–19.2 Ma.

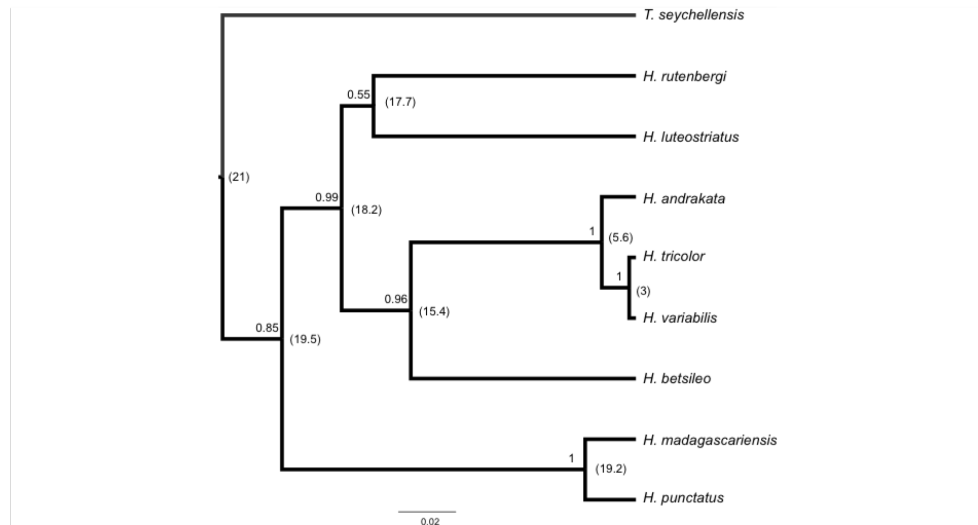

Fig. 1. Bayesian species tree of the relationships between *Tachycnemis* and *Heterixalus* inferred using the multispecies coalescent in *BEAST. Numbers on branches are Bayesian posterior probabilities. The red branch indicates the placement of *T. seychellensis* whereas those of *Heterixalus* spp. are black. Numbers in parentheses at nodes are mean *p*-distances for *cytb* between two lineages. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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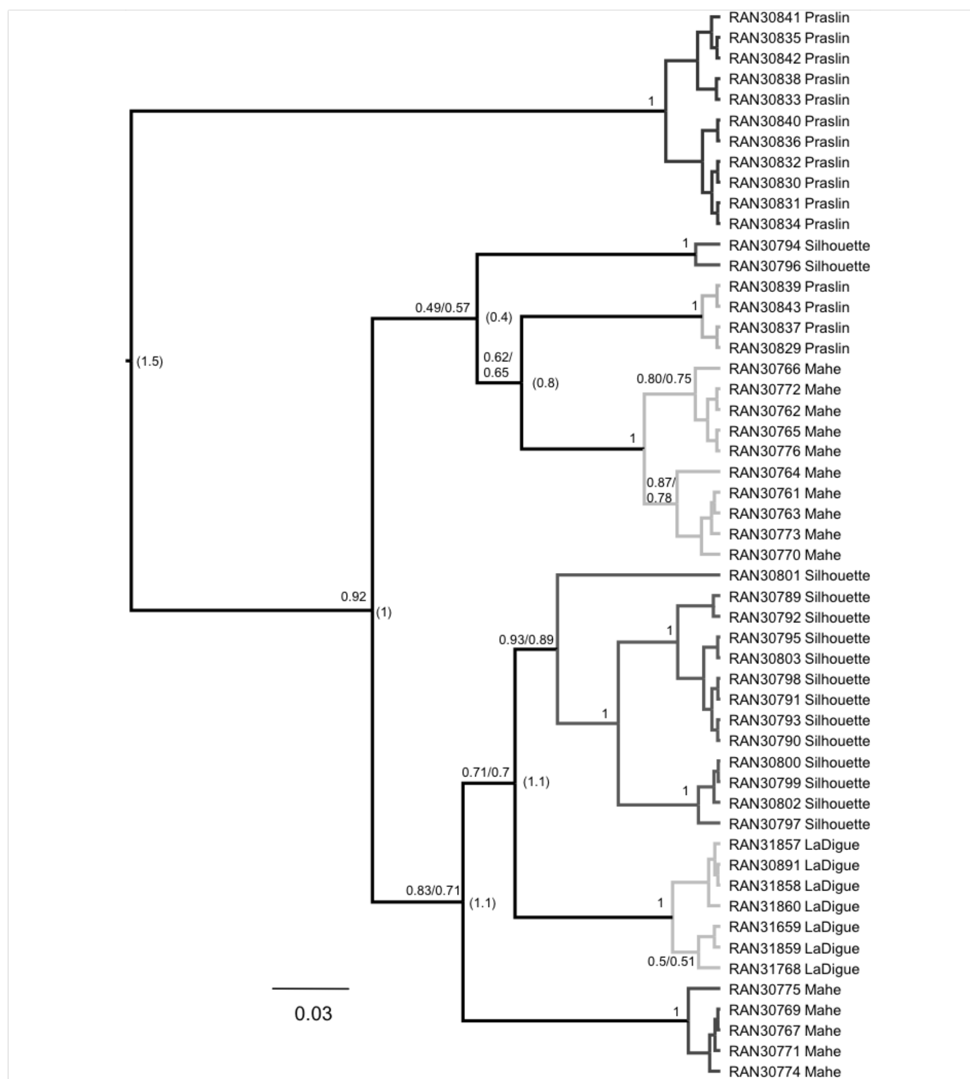

Fig. 2. Bayesian inference tree for *Tachycnemis seychellensis* using three mtDNA gene fragments (*16s*, *cytb*, *cox1*) analysed with the BEAST software package. Numbers on branches are Bayesian posterior probabilities under AIC/BIC; when a single number is used both AIC and BIC schemes produced the same BPPs. Clade colours refer to those used in Fig. 3. Numbers in parentheses at nodes are mean *p*-distances for *cytb* between two lineages.

3.2. Genetic variation within T. seychellensis

We aligned three mitochondrial genes for the 52 specimens: *16s* consisted of 599 bp with three variable sites (v.s.), all of which were parsimony-informative (p.s.); *cytb* 763 bp (32 v.s., 30 p.s.); and *cox1* 786 bp (16 v.s., 13 p.s.). The dataset was almost complete, with very little missing sequence data across all genes and no genes missing for any individual. No saturation was detected. The *rag1* and *tyr* data were constant in 20 and 19 samples sampled across all populations, respectively. The *pomc* data consisted of 629 bp (7 v.s., 6 p.s.) for 50 specimens; and *rho* 337 bp (2 v.s., 2 p.s.) for 25 specimens. The sample size for *pomc* and *rho* was reduced because of a shorter amplified sequence length of some samples and because of a lack of

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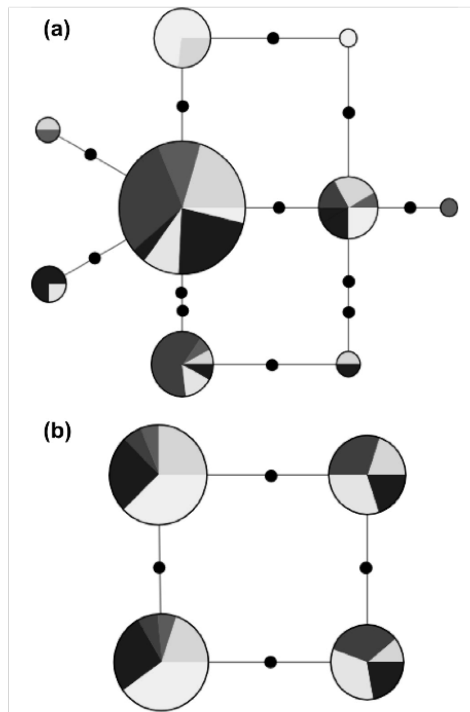

Fig. 3. Median-joining haplotype networks for two nuDNA genes for *Tachycnemis seychellensis* determined using NETWORK: (a) *pomc*; (b) *rho*. Segment colours refer to clades in the mtDNA phylogenetic tree (Fig. 2). Black circles on connecting branches indicate the number of mutational steps between haplotypes.

confidence in the accuracy of the PHASE calling of the small number of variable sites.

The best partitioning strategies and models as determined by PartitionFinder for the mtDNA analyses were the same under AIC and AICc but different under BIC (Table 2). Thus, two BEAST analyses were run under these alternatives, and the resulting tree topologies were identical and support values nearly so (Fig. 2).

The mtDNA has a maximum *p*-distance of 1.5% between any of the seven haplotype groups, and no simple geographic structure is observed in the mtDNA tree (Fig. 3), with samples from all islands except La Digue comprising two haplotype clades that are not sister groups, although not all relationships are well supported. The mean *p*-distance for *cytb* among the main mtDNA haplotype clades ranges from 0.4% to 1.5% (Fig. 2). Given an approximate rate of 0.6–1% per million years for *cytb* in amphibians (see Elmer et al., 2007), this marker suggests that extant mtDNA haplotype lineages of *T. seychellensis* began diverging approximately 0.75–1.25 Ma.

The two variable nuclear genes yielded networks with a general lack of geographic structure (Fig. 3). For *pomc* (Fig. 3a) there is a small amount of population structuring, with endemic haplotypes shared by multiple individuals within Praslin and Mahé. For *rho* (Fig. 3b) each of the four haplotypes are found on all islands except La Digue (two haplotypes).

Fu's *Fs* results indicate recent expansions for all of the island populations with maximal significance (Table 4). Tajima's *D* values suggest an opposite trend, with positive values indicating either a population size decrease or balancing selection, but Tajima's *D* results are not significant for any island (Table 4).

Table 4
Population genetic statistics for Fu's *Fs* and Tajima's *D* for mtDNA data for 52 *Tachycnemis seychellensis*.

Island	*N*	*Fs*	*p*-Values	Tajima's D	*p*-Values
Mahé	15	−8.99022	0.00000	1.59096	0.96300
Silhouette	15	−11.24523	0.00000	0.24311	0.62500
Praslin	15	−10.71708	0.00000	1.52485	0.95600
La Digue	7	−9.21700	0.00000	0.20619	0.65400

4. Discussion

4.1. Monophyly of Heterixalus?

It has been estimated that *T. seychellensis* diverged from its closest living relative in Madagascar 9.79–35.34 Ma (Crottini et al., 2012), which implies transoceanic dispersal to the Seychelles given that this microcontinent split from Madagascar approximately 84 Ma (Ali and Aitchison, 2008; Plummer and Belle, 1995) and India by 64 Ma (McKenzie and Sclater, 1971; Norton and Sclater, 1979). Transoceanic dispersal remains a rarely documented phenomenon in amphibians, but see Hedges et al. (1992), Measey et al. (2007), Vences et al. (2003a,b, 2004).

Our analyses using the multispecies coalescent support previous studies based on concatenated multilocus DNA sequence data (e.g. Pyron and Wiens, 2011; Vences et al., 2003a,b; Wollenberg et al. 2007) that have hypothesised *T. seychellensis* to be the sister taxon to *Heterixalus*. Translation of *cytb p*-distances to divergence times among lineages produces estimates that fall within Crottini et al.'s (2012) estimate of 9.79–35.34 Ma for the divergence between *T. seychellensis* and its closest living relative. The *cytb p*-distances between *Tachycnemis* and *Heterixalus* spp. (Fig. 1) are clearly more in agreement with overseas dispersal than Seychelles-Africa or Seychelles-Madagascar Cretaceous vicariance as an explanation for the origin of *Tachycnemis* in the Seychelles.

4.2. Genetic variation within T. seychellensis

The results of our genetic analyses are consistent with Nussbaum and Wu's (1995) interpretation that the Seychelles treefrog represents a single species. The low levels of genetic diversity within *T. seychellensis* and lack of notable phylogeographic structure can be explained by a rapid range expansion (supported by results for Fu's *Fs*) and/or by multiple admixture events possibly during eustatic sea-level fluctuations. The latter is plausible given that all island populations, apart from La Digue, have multiple mtDNA haplotype clades and that nuDNA haplotypes show no clear geographic structuring. The relatively low levels of genetic variation within the Seychelles treefrog are comparable with several other Seychelles taxa such as *Drosophila* flies (Legrand et al., 2009) and freshwater turtles (Silva et al., 2010), although the turtles are probably a recent human introduction (Fritz et al., 2013). Conversely, studies of other taxa including lizards (Rocha et al., 2010a,b, 2011, 2013; Valente et al., 2014), a freshwater crab (Daniels, 2011), and a sooglossid frog (Taylor et al., 2012) have revealed much higher levels of inter-island genetic variation. It remains to be fully assessed whether differences in patterns of genetic variation among Seychelles organisms can be explained by ecology (and dispersal ability) and/or duration of residency. The presence of low genetic diversity and little phylogeographic

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structure but high morphological variation as is observed in *T. seychellensis* is unusual in (at least adult) anurans and it is difficult to find any examples in the literature (though see e.g., Gvoždík et al., 2008, 2010).

The combination of low levels of genetic diversity (and little phylogeographic structure) within *T. seychellensis* yet substantial morphological variation is perhaps best explained by rapid local adaptation to different environmental settings, ecophenotypic plasticity, or from previous genetic bottlenecks and/or continuing small population sizes (see also Nussbaum and Wu, 1995). The latter explanation seems unlikely on the islands of Mahé and Praslin where *T. seychellensis* is abundant in the sampled populations (STM, RAN, DJG *pers. obs.*). These explanations could be tested using population-genetic analyses of data from more rapidly evolving nuclear markers.

Genetic (*cytb p-*) distances between populations of *T. seychellensis* on different islands (see Fig. 2) provide no evidence for admixture between the islands after 200–333 ka. This might suggest that during the most recent sea-level fluctuations (~10 ka), where all islands would have been connected (Colonna et al., 1996; Rohling et al., 1998; Siddall et al., 2003; Camoin et al., 2004; Miller et al., 2005), little migration occurred or, if migration did occur, mitochondrial haplotypes did not become fixed.

5. Conclusions

We find support for the sister-group relationship between *T. seychellensis* and a monophyletic *Heterixalus*. There is little genetic variation within *T. seychellensis*, even among populations on the four different islands within its range, and the variation is not strongly spatially structured. This is consistent with Nussbaum and Wu's (1995) interpretation that there is a single species of Seychelles treefrog. The patterns of genetic variation that we have discovered do not allow us to reject Nussbaum and Wu's (1995) proposal that substantial morphological variation within *T. seychellensis* is the result of local ecological adaptation and/or small population sizes now and/or in the past, though ecophenotypic plasticity might also be considered.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.02.004.

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APPENDIX 3

Specimens used for morphological analyses in Chapter 3

Grandisonia alternans

Félicite
UMMZ 167806, UMMZ 167807, UMMZ 167810, UMMZ 167813, UMMZ 167818, UMMZ 167821, UMMZ 167822.

Frégate
UMMZ 145041, UMMZ 145043, UMMZ 145044, UMMZ 145045, UMMZ 145048, UMMZ 145049, UMMZ 145051, UMMZ 145054, UMMZ 145055, UMMZ 145057, UMMZ 145058, UMMZ 145059, UMMZ 145060, UMMZ 145062, UMMZ 145067, UMMZ 145071, UMMZ 145258, UMMZ 175296, UMMZ 175297, UMMZ 175304, UMMZ 175307, UMMZ 177194, UMMZ 177197, UMMZ 177198, UMMZ 177201, UMMZ 189181, UMMZ 189182, UMMZ 189183.

La Digue
MW 10442, MW 10447, UMMZ 145143, UMMZ 145164, UMMZ 145188.

Mahé
MW 09507, MW 10465, MW 10474, MW 10479, MW 10557, MW 10558, MW 10569, MW 10570, MW 10576, MW 7762, UMMZ 145219, UMMZ 145970, UMMZ 175431, UMMZ 177188, UMMZ 177189, UMMZ 179959, UMMZ 179960, UMMZ 181067, UMMZ 183015, UMMZ 192939, UMMZ 200517, UMMZ 200518, UMMZ 200519, UMMZ 200575,

Praslin
MW 07759, MW 10271, MW 10428.

Silhouette
UMMZ 193113, UMMZ 193157, UMMZ 179961, UMMZ 179965, UMMZ 179967, UMMZ 179979, UMMZ 179983, UMMZ 179984, UMMZ 179985, UMMZ 179987, UMMZ 179992, UMMZ 180003, UMMZ 180004, UMMZ 180005, UMMZ 180012, UMMZ 180013, UMMZ 180014, UMMZ 180015, UMMZ 180017, UMMZ 180031, UMMZ 180032, UMMZ 180035, UMMZ 180036, UMMZ 180037, UMMZ 180038, UMMZ 180052, UMMZ 180053, UMMZ 180054, UMMZ 180055, UMMZ 180057, UMMZ 180060, UMMZ 193113, UMMZ 193127, UMMZ 193141.

Grandisonia larvata

Félicite
UMMZ 167812, UMMZ 167816, UMMZ 167819, UMMZ 167820, UMMZ 167823, UMMZ 167824, UMMZ 167825, UMMZ 167826.

La Digue
UMMZ 145124, UMMZ 145126, UMMZ 145129, UMMZ 145132, UMMZ 145183, UMMZ 145184, UMMZ 145185, UMMZ 145186, UMMZ 145189, UMMZ 145190, UMMZ 145191, UMMZ 145194, UMMZ 145195, UMMZ 145196, UMMZ 145197.

Mahé
UMMZ 145036, UMMZ 177136, UMMZ 177138, UMMZ 177142, UMMZ 177163, UMMZ 177164, UMMZ 177172, UMMZ 177174, UMMZ 177177, UMMZ 180858, UMMZ 181624, UMMZ 188612, UMMZ 188613, UMMZ 188614, UMMZ 188615.

Praslin
UMMZ 167798, UMMZ 200526, UMMZ 200528, UMMZ 200529.

Silhouette
MW 10015, UMMZ 146010, UMMZ 146058, UMMZ 167881, UMMZ 167882, UMMZ 180141.

Ste Anne
UMMZ 145087, UMMZ 146730, UMMZ 146798, UMMZ 175308.

Grandisonia sechellensis

Mahé
UMMZ 145135, UMMZ 150950, UMMZ 167802, UMMZ 175424, UMMZ 175428, UMMZ 175433, UMMZ 175438, UMMZ 175440, UMMZ 175441, UMMZ 177124, UMMZ 177186, UMMZ 177187, UMMZ 181680, UMMZ 182821, UMMZ 182823, UMMZ 192975, UMMZ 193233, UMMZ 200536, UMMZ 200537, UMMZ 200574.

Praslin
MW 10018, MW 10269, MW 10450, UMMZ 145122.

Silhouette
UMMZ 167834, UMMZ 167857, UMMZ 167861, UMMZ 167872, UMMZ 167878, UMMZ 179972, UMMZ 179974, UMMZ 180080, UMMZ 180083, UMMZ 180084, UMMZ 180086, UMMZ 180087, UMMZ 180135, UMMZ 180136, UMMZ 180137, UMMZ 180138, UMMZ 180140, UMMZ 180144, UMMZ 180145, UMMZ 180146.

Hypogeophis brevis

BMNH 1910.3.8.85, BMNH 1946.3.18.84, MW 10001, MW 10005, MW 10006, MW 10022, MW 10468, MW 10469, MW 10470, MW 10475, MW 10476, MW 10477, MW 10478, MW 10483, MW 10486, MW 9509, UMMZ 145023, UMMZ 145039, UMMZ 145180, UMMZ 146295, UMMZ 168115, UMMZ 175435, UMMZ 175493, UMMZ 180852, UMMZ 180853, UMMZ 180854, UMMZ 180855, UMMZ 181384, UMMZ 181385, UMMZ 182998, UMMZ 182999, UMMZ 183066, UMMZ 189440, UMMZ 189441, UMMZ 189442, UMMZ 192977, UMMZ 193089, UMMZ 200520, UMMZ 221084.

***Hypogeophis* cf. *brevis* CR**

MW 10023, MW 10024, MW 10566, MW 10567, MW 10568.

***Hypogeophis* cf. *brevis* Praslin**

BMNH 1987.2109, MW 10021, MW 10278, MW 10420, MW 10424, MW 10425, MW 10426, MW 10427, MW 10429, MW 10430, MW 10431, MW 10454, MW 10455, MW 10456, MW 10457.

Hypogeophis rostratus

Curieuse
UMMZ 167793, UMMZ 167794, UMMZ 167795, UMMZ 167796, UMMZ 167797, UMMZ 200497.

Félicite
UMMZ 167719, UMMZ 167721, UMMZ 167722, UMMZ 167723, UMMZ 167724, UMMZ 167725, UMMZ 167729, UMMZ 167730, UMMZ 167731, UMMZ 167743, UMMZ 167744, UMMZ 167745, UMMZ 167746, UMMZ 167747, UMMZ 167748, UMMZ 167749, UMMZ 167750, UMMZ 167751, UMMZ 167752, UMMZ 167808.

Frégate
UMMZ 145228, UMMZ 145229, UMMZ 145230, UMMZ 145231, UMMZ 145232, UMMZ 145234, UMMZ 145235, UMMZ 145240, UMMZ 145241, UMMZ 145242, UMMZ 145243, UMMZ 145244, UMMZ 145246, UMMZ 145247, UMMZ 145249, UMMZ 145250, UMMZ 145252, UMMZ 145253, UMMZ 145255, UMMZ 145259, UMMZ 145262, UMMZ 145263, UMMZ 145264, UMMZ 145265, UMMZ 145266, UMMZ 145269, UMMZ 145272, UMMZ 145273, UMMZ 175316, UMMZ 175317, UMMZ 175319, UMMZ 175320, UMMZ 175321, UMMZ 175322, UMMZ 177446, UMMZ 177451, UMMZ 190464, UMMZ 190465, UMMZ 20046.

La Digue
UMMZ 145364, UMMZ 145365, UMMZ 145366, UMMZ 145367, UMMZ 145368, UMMZ 145369, UMMZ 145371, UMMZ 145373, UMMZ 145374, UMMZ 145375, UMMZ 145376, UMMZ 145377, UMMZ 145378, UMMZ 145379, UMMZ 145380, UMMZ 145381, UMMZ 145382, UMMZ 145383, UMMZ 145384, UMMZ 145385, UMMZ 145386, UMMZ 145387, UMMZ 145388, UMMZ 145389, UMMZ 145390, UMMZ 145531, UMMZ 145532, UMMZ 145533, UMMZ 145534, UMMZ 145535, UMMZ 145536, UMMZ 145537, UMMZ 145538, UMMZ 145539, UMMZ 145540, UMMZ 145541, UMMZ 145542, UMMZ 145542, UMMZ 145543, UMMZ 145546, UMMZ 145547, UMMZ 145548, UMMZ 145549, UMMZ 145550, UMMZ 145551, UMMZ 145552.

Mahé
UMMZ 167754, UMMZ 167755, UMMZ 175447, UMMZ 175448, UMMZ 175449, UMMZ 175452, UMMZ 175453, UMMZ 175456, UMMZ 175457, UMMZ 175458, UMMZ 177225, UMMZ 177227, UMMZ 177228, UMMZ 177229, UMMZ 177230, UMMZ 177256, UMMZ 177327, UMMZ 177332, UMMZ 177334, UMMZ 177336, UMMZ 177337, UMMZ 177338, UMMZ 177339, UMMZ 177341, UMMZ 177343, UMMZ 177344, UMMZ 177345, UMMZ 177349, UMMZ 177352, UMMZ 177355, UMMZ 177356, UMMZ 177357, UMMZ 177359, UMMZ 177360, UMMZ 177363, UMMZ 177440, UMMZ 177441, UMMZ 177442, UMMZ 177443, UMMZ 177448, UMMZ 177449, UMMZ 177477, UMMZ 179805, UMMZ 179811, UMMZ 179812, UMMZ 179818, UMMZ 179819, UMMZ 179821, UMMZ 179822, UMMZ 179823, UMMZ 180645, UMMZ 180646, UMMZ 180647, UMMZ 180648, UMMZ 180650, UMMZ 180654, UMMZ 180665, UMMZ 180666, UMMZ 180670, UMMZ 180672, UMMZ 182822, UMMZ 182922, UMMZ 182926, UMMZ 182930, UMMZ 182931, UMMZ 182933, UMMZ 182934, UMMZ 182937, UMMZ 182939, UMMZ 182941, UMMZ 182942, UMMZ 183019, UMMZ 183020, UMMZ 183022, UMMZ 192970, UMMZ 193026, UMMZ 193027, UMMZ 193028, UMMZ 193029, UMMZ 193030, UMMZ 193031, UMMZ 193032, UMMZ 193033, UMMZ 193034, UMMZ 193035, UMMZ 193036, UMMZ 193037, UMMZ 193038, UMMZ 193039, UMMZ 193040, UMMZ 193041, UMMZ 193042, UMMZ 193043, UMMZ 193044, UMMZ 193062, UMMZ 193063, UMMZ 193064, UMMZ 193065, UMMZ 193066, UMMZ 193067, UMMZ 193068, UMMZ 193069, UMMZ 193070, UMMZ 193071, UMMZ 195773, UMMZ 195783.

Praslin
UMMZ 145275, UMMZ 145277, UMMZ 145284, UMMZ 145285, UMMZ 145286, UMMZ 145287, UMMZ 145288, UMMZ 145289, UMMZ 145290, UMMZ 145291, UMMZ 145292, UMMZ 145293, UMMZ 145294, UMMZ 145295, UMMZ 145298, UMMZ 145299, UMMZ 145300, UMMZ 145325, UMMZ 145326, UMMZ 145330, UMMZ 145331, UMMZ 145332, UMMZ 145334, UMMZ 145335, UMMZ 145336, UMMZ 145340, UMMZ 145342, UMMZ 145344, UMMZ 145347, UMMZ 145348, UMMZ 145351, UMMZ 145571, UMMZ 175327, UMMZ 175328, UMMZ 175329, UMMZ 175330, UMMZ 175331, UMMZ 175332, UMMZ 175371, UMMZ 175373, UMMZ 175383.

Silhouette
UMMZ 146074, UMMZ 146107, UMMZ 146108, UMMZ 146109, UMMZ 167758, UMMZ 167760, UMMZ 167761, UMMZ 167762, UMMZ 167763, UMMZ 167764, UMMZ 167765, UMMZ 167787, UMMZ 167788, UMMZ 167789, UMMZ 167790, UMMZ 167791, UMMZ 179842, UMMZ 179844, UMMZ 179845, UMMZ 179846, UMMZ 179847, UMMZ 179848, UMMZ 179853, UMMZ 179854, UMMZ 179855, UMMZ 179856, UMMZ 179857, UMMZ 179859, UMMZ 179878, UMMZ 179880, UMMZ 179882, UMMZ 179883, UMMZ 179884, UMMZ 179885, UMMZ 179886, UMMZ 179887, UMMZ 179889, UMMZ 179890, UMMZ 179891, UMMZ 179892, UMMZ 179893.

Ste Anne
UMMZ 167702, UMMZ 167703, UMMZ 167704, UMMZ 167706, UMMZ 167707, UMMZ 167708, UMMZ 167709, UMMZ 167710, UMMZ 200437, UMMZ 200440, UMMZ 200441, UMMZ 200442, UMMZ 200443, UMMZ 200446, UMMZ 200447, UMMZ 200461, UMMZ 200462.

Praslinia cooperi

Mahé
UMMZ 177460, UMMZ 183006, UMMZ 192934, UMMZ 192935, UMMZ 192936, UMMZ 193019, UMMZ 195775, UMMZ 200565.

Silhouette
UMMZ 179958, UMMZ 189437, UMMZ 192894, UMMZ 192930, UMMZ 192931, UMMZ 192932, UMMZ 192933.

APPENDIX 4

Details of samples used in Chapter 4

Details of samples used and sequence data generated in Chapter 4. Museum code SM refers to samples collected during this thesis and UMMZ refers to samples housed at the University of Michigan Museum of Zoology. An "X" symbol indicates that novel sequences were generated.

Species	Museum	Sample ID	Island	Locality	*cytb*	*bdnf*	*brev5*	*pomc*	*rost5*
G. alternans	UMMZ	31051	Fregate	Settlement Plateau	X	X	X	X	X
G. alternans	UMMZ	31052	Fregate	Settlement Plateau	X	X		X	X
G. alternans	UMMZ	31053	Fregate	Settlement Plateau	X	X	X	X	X
G. alternans	UMMZ	31054	Fregate	Settlement Plateau	X	X	X	X	X
G. alternans	UMMZ	31055	Fregate	Settlement Plateau	X	X	X	X	X
G. alternans	UMMZ	31056	Fregate	Settlement Plateau	X	X	X	X	X
G. alternans	UMMZ	31057	Fregate	Settlement Plateau	X				
G. alternans	UMMZ	31058	Fregate	Settlement Plateau	X				
G. alternans	UMMZ	31059	Fregate	Settlement Plateau	X				
G. alternans	UMMZ	31060	Fregate	Settlement Plateau	X				
G. alternans	UMMZ	31061	Fregate	Settlement Plateau	X				
G. alternans	UMMZ	31062	Fregate	Settlement Plateau	X	X	X	X	X
G. alternans	UMMZ	31063	Fregate	Settlement Plateau	X				
G. alternans	UMMZ	31064	Fregate	Settlement Plateau	X				
G. alternans	UMMZ	31065	Fregate	Settlement Plateau	X				
G. alternans	UMMZ	31066	Fregate	Settlement Plateau	X				
G. alternans	UMMZ	31067	Fregate	Settlement Plateau	X				
G. alternans	UMMZ	31074	Fregate	Settlement Plateau	X				
G. alternans	SM	318	La Digue	Top of road to Grand Anse	X	X		X	X
G. alternans	SM	323	La Digue	Top of road to Grand Anse	X	X	X	X	X
G. alternans	SM	324	La Digue	Top of road to Grand Anse	X	X	X	X	X
G. alternans	SM	325	La Digue	Top of road to Grand Anse	X	X	X	X	X

G. alternans	SM	329	La Digue	Top of road to Grand Anse	X	X	X	X	X
G. alternans	SM	181	Mahe	Case Dent	X	X	X		X
G. alternans	SM	185	Mahe	Mare Aux Cochon	X	X	X	X	X
G. alternans	SM	186	Mahe	Mare Aux Cochon	X	X	X	X	X
G. alternans	SM	343	Mahe	Bamboo CR trail	X	X	X		X
G. alternans	SM	350	Mahe	Bamboo CR trail	X	X	X	X	X
G. alternans	SM	352	Mahe	Bamboo CR trail	X				
G. alternans	SM	372	Mahe	Stream La Reserve	X	X	X	X	
G. alternans	SM	374	Mahe	La Reserve	X	X	X	X	X
G. alternans	SM	377	Mahe	La Reserve	X	X	X	X	X
G. alternans	SM	384	Mahe	Congo Rouge - below Pitcher Plants	X	X	X	X	X
G. alternans	UMMZ	31314	Mahe	Grand Bois River	X	X	X	X	X
G. alternans	SM	130	Praslin	Vallee De Mai	X	X	X	X	X
G. alternans	SM	131	Praslin	Vallee De Mai	X	X	X	X	X
G. alternans	SM	296	Praslin	Fond Peper	X	X	X	X	X
G. alternans	SM	297	Praslin	Fond Peper	X	X	X	X	X
G. alternans	SM	303	Praslin	Fond Peper	X	X	X	X	X
G. alternans	SM	480	Praslin	Fond Peper	X	X	X	X	X
G. alternans	SM	397	Silhouette	Mare Aux Cochons	X	X	X	X	X
G. alternans	SM	398	Silhouette	Mare Aux Cochons	X	X	X	X	X
G. alternans	SM	417	Silhouette	Mare Aux Cochons	X	X	X	X	X
G. alternans	SM	418	Silhouette	Mare Aux Cochons	X	X	X	X	X
G. alternans	UMMZ	31133	Silhouette	Gratte Fess Grand Barbe Side	X	X	X	X	X
G. alternans	UMMZ	31134	Silhouette	Gratte Fess Grand Barbe Side	X	X	X	X	X
G. alternans	UMMZ	31135	Silhouette	Gratte Fess Grand Barbe Side	X	X	X	X	X
G. alternans	UMMZ	31138	Silhouette	Gratte Fess Grand Barbe Side	X	X	X	X	X
G. alternans	UMMZ	31139	Silhouette	Gratte Fess Grand Barbe Side	X				
G. alternans	UMMZ	31235	Silhouette	Gratte Fess La Passe Side	X	X	X	X	X
G. alternans	UMMZ	31236	Silhouette	Gratte Fess La Passe Side	X				
G. alternans	UMMZ	31237	Silhouette	Gratte Fess La Passe Side	X				
G. alternans	UMMZ	31238	Silhouette	Gratte Fess La Passe Side	X				
G. alternans	UMMZ	31242	Silhouette	Gratte Fess La Passe Side	X	X	X	X	X
G. alternans	UMMZ	31243	Silhouette	Gratte Fess La Passe Side	X				
G. alternans	UMMZ	31244	Silhouette	Gratte Fess La Passe Side	X				

G. alternans	UMMZ	31245	Silhouette	Gratte Fess La Passe Side	X				
G. alternans	UMMZ	31247	Silhouette	Jardin Marron Trail Coco-De-Nen	X				
G. alternans	UMMZ	31301	Silhouette	Grand Barbe Side of Gratte Fess Trail	X				
G. alternans	UMMZ	31349	Silhouette	Gratte Fess Trail	X				
G. alternans	UMMZ	31351	Silhouette	Gratte Fess Trail	X				
G. alternans	UMMZ	31352	Silhouette	Gratte Fess Trail	X				
G. alternans	UMMZ	31353	Silhouette	Gratte Fess Trail	X				
G. alternans	UMMZ	31354	Silhouette	Gratte Fess Trail	X				
G. alternans	UMMZ	31355	Silhouette	Gratte Fess Trail	X				
G. alternans	UMMZ	31356	Silhouette	Gratte Fess Trail	X				
G. alternans	UMMZ	31357	Silhouette	Gratte Fess Trail	X				
G. alternans	UMMZ	31358	Silhouette	Gratte Fess Trail	X				
G. alternans	UMMZ	31395	Silhouette	La Passe Side of Gratte Fess Trail	X				
G. alternans	UMMZ	31468	Silhouette	Grand Barbe Side of Gratte Fess Trail	X				
G. alternans	UMMZ	31472	Silhouette	Grand Barbe Side of Gratte Fess Trail	X				
G. alternans	UMMZ	31473	Silhouette	Grand Barbe Side of Gratte Fess Trail	X				
G. alternans	UMMZ	31477	Silhouette	Grand Barbe Side of Gratte Fess Trail	X				
G. larvata	UMMZ	25168	La Digue	Behind La Di Passe	X				
G. larvata	UMMZ	31187	La Digue	Plateau at Reunion	X	X	X	X	X
G. larvata	UMMZ	31188	La Digue	Plateau at Reunion	X	X	X	X	X
G. larvata	UMMZ	31189	La Digue	Plateau at Reunion	X	X	X		X
G. larvata	UMMZ	31190	La Digue	Plateau at Reunion	X	X	X	X	X
G. larvata	UMMZ	31191	La Digue	Plateau at Reunion	X	X	X	X	X
G. larvata	UMMZ	31192	La Digue	Plateau at Reunion	X				
G. larvata	UMMZ	31674	La Digue	Anse Reunion	X				
G. larvata	UMMZ	31828	La Digue	Anse Reunion	X				
G. larvata	UMMZ	31829	La Digue	Anse Reunion	X				
G. larvata	UMMZ	31830	La Digue	Anse Reunion	X				
G. larvata	UMMZ	31831	La Digue	Anse Reunion	X				
G. larvata	UMMZ	31832	La Digue	Anse Reunion	X				
G. larvata	UMMZ	31833	La Digue	Anse Reunion	X				
G. larvata	UMMZ	31834	La Digue	Anse Reunion	X				
G. larvata	SM	351	Mahe	Les Cannelles	X	X	X	X	
G. larvata	SM	360	Mahe	Chemin Dame Le Roi	X	X	X		X

G. larvata	SM	361	Mahe	Chemin Dame Le Roi	X	X	X	X	X
G. larvata	SM	362	Mahe	Chemin Dame Le Roi	X	X	X	X	
G. larvata	SM	380	Mahe	Stream La Reserve	X	X	X	X	X
G. larvata	UMMZ	31201	Mahe	Bel Ombre	X	X	X	X	X
G. larvata	UMMZ	31202	Mahe	Bel Ombre	X	X	X	X	
G. larvata	UMMZ	31203	Mahe	Bel Ombre	X	X	X	X	X
G. larvata	SM	132	Praslin	Fond Peper	X	X	X	X	X
G. larvata	SM	281	Praslin	Fond Peper	X				X
G. larvata	SM	328	Praslin	Above Fond Peper	X	X	X	X	X
G. larvata	SM	473	Praslin	Zimbabwe	X	X	X	X	X
G. larvata	SM	474	Praslin	Zimbabwe	X	X	X	X	X
G. larvata	UMMZ	31760	Praslin	Between Vallee de Mai and Baie Ste Anne	X	X	X	X	X
G. larvata	UMMZ	31761	Praslin	Between Vallee de Mai and Baie Ste Anne	X	X	X	X	X
G. larvata	UMMZ	31598	Ste Anne	SW side of island	X	X	X	X	
G. larvata	SM	231	Silhouette	Anse Mordon trail	X		X		
G. larvata	SM	238	Silhouette	Anse Mordon trail	X	X	X	X	X
G. larvata	SM	239	Silhouette	Anse Mordon trail	X	X	X	X	X
G. larvata	SM	240	Silhouette	Anse Mordon trail	X	X	X	X	X
G. larvata	SM	241	Silhouette	Anse Mordon trail	X				
G. larvata	SM	250	Silhouette	Grand Barbe	X	X	X	X	X
G. larvata	SM	411	Silhouette	Mare Aux Cochons	X	X	X	X	X
G. larvata	SM	412	Silhouette	Mare Aux Cochons	X	X	X	X	X
G. larvata	UMMZ	31364	Silhouette	Gratte Fess Trail	X	X	X	X	X
G. sechellensis	SM	164	Mahe	Mare Aux Cochon	X	X	X	X	X
G. sechellensis	SM	187	Mahe	Mare Aux Cochon	X				
G. sechellensis	SM	190	Mahe	Casse Dent, Grand Bois River	X				
G. sechellensis	SM	194	Mahe	Mare Aux Cochon	X				
G. sechellensis	SM	370	Mahe	La Reserve	X	X	X	X	X
G. sechellensis	SM	371	Mahe	La Reserve	X	X	X		
G. sechellensis	SM	375	Mahe	La Reserve	X	X	X	X	X
G. sechellensis	SM	383	Mahe	Congo Rouge - before Pictcher's	X	X			X
G. sechellensis	UMMZ	25768	Mahe	Between Mt. Simpson Estate and Mare Aux Cochon	X				
G. sechellensis	UMMZ	25769	Mahe	Between Mt. Simpson Estate and Mare Aux	X				

				Cochon					
G. sechellensis	UMMZ	26802	Mahe	Between Mt. Simpson Estate and Mare Aux Cochon	X				
G. sechellensis	UMMZ	26803	Mahe	Between Mt. Simpson Estate and Mare Aux Cochon	X				
G. sechellensis	UMMZ	26804	Mahe	Between Mt. Simpson Estate and Mare Aux Cochon	X				
G. sechellensis	UMMZ	26886	Mahe	Grand Bois River	X				
G. sechellensis	UMMZ	26887	Mahe	Grand Bois River	X				
G. sechellensis	UMMZ	31312	Mahe	Mt. Coton	X		X	X	
G. sechellensis	UMMZ	31323	Mahe	Grand Bois River	X		X	X	
G. sechellensis	UMMZ	31387	Mahe	Grand Bois River	X	X	X	X	X
G. sechellensis	UMMZ	31389	Mahe	Grand Bois River	X	X	X	X	X
G. sechellensis	UMMZ	31390	Mahe	Grand Bois River	X	X	X	X	X
G. sechellensis	UMMZ	31600	Mahe	Grand Bois River	X	X	X	X	X
G. sechellensis	UMMZ	31602	Mahe	Grand Bois River	X	X	X	X	X
G. sechellensis	UMMZ	31629	Mahe	Grand St Louis River	X	X	X	X	X
G. sechellensis	UMMZ	34279	Mahe	Grand Bois River	X				
G. sechellensis	SM	278	Praslin	Fond Peper	X	X	X	X	X
G. sechellensis	SM	298	Praslin	Fond Peper	X	X	X	X	X
G. sechellensis	SM	327	Praslin	Above Fond Peper	X	X	X	X	X
G. sechellensis	SM	478	Praslin	Vallee De Mai	X			X	
G. sechellensis	SM	210	Silhouette	Jardin Maron	X				
G. sechellensis	SM	248	Silhouette	Grand Barbe	X	X	X	X	X
G. sechellensis	SM	249	Silhouette	Grand Barbe	X	X	X	X	X
G. sechellensis	UMMZ	31249	Silhouette	Jardin Maron	X	X	X	X	
G. sechellensis	UMMZ	31379	Silhouette	Grand Barbe	X	X	X	X	
G. sechellensis	UMMZ	31380	Silhouette	Grand Barbe	X	X	X	X	X
G. sechellensis	UMMZ	31381	Silhouette	Grand Barbe	X	X	X	X	X
G. sechellensis	UMMZ	31382	Silhouette	Grand Barbe	X				
G. sechellensis	UMMZ	31383	Silhouette	Grand Barbe	X				
G. sechellensis	UMMZ	31384	Silhouette	Grand Barbe	X				
G. sechellensis	UMMZ	31385	Silhouette	Grand Barbe	X				
G. sechellensis	UMMZ	31453	Silhouette	Grand Barbe	X	X	X	X	X
G. sechellensis	UMMZ	31486	Silhouette	Grand Barbe	X	X	X	X	X

G. sechellensis	UMMZ	31488	Silhouette	Grand Barbe	X				
G. sechellensis	UMMZ	31489	Silhouette	Grand Barbe	X				
G. sechellensis	UMMZ	31490	Silhouette	Grand Barbe	X				
G. sechellensis	UMMZ	31491	Silhouette	Grand Barbe	X				
G. sechellensis	UMMZ	31492	Silhouette	Grand Barbe	X				
G. sechellensis	UMMZ	31494	Silhouette	Grand Barbe	X				
G. sechellensis	UMMZ	31495	Silhouette	Grand Barbe	X				
G. sechellensis	UMMZ	31496	Silhouette	Grand Barbe	X				
G. sechellensis	UMMZ	31497	Silhouette	Grand Barbe	X				
H. brevis	SM	162	Mahe	La Reserve	X	X	X	X	X
H. brevis	SM	163	Mahe	Grand Bois River	X	X	X	X	X
H. brevis	SM	192	Mahe	Case Dent	X	X	X	X	
H. brevis	SM	193	Mahe	Mare Aux Cochons	X	X	X	X	X
H. brevis	SM	283	Mahe	La Reserve	X	X	X		X
H. brevis	SM	354	Mahe	Bamboo on Congo Rouge trail	X	X	X	X	X
H. brevis	SM	355	Mahe	Bamboo on Congo Rouge trail	X	X	X	X	X
H. brevis	SM	356	Mahe	Bamboo on Congo Rouge trail	X	X	X	X	X
H. brevis	SM	366	Mahe	La Reserve	X	X	X	X	
H. brevis	SM	367	Mahe	La Reserve	X	X	X	X	X
H. brevis	SM	368	Mahe	La Reserve	X	X	X	X	X
H. brevis	SM	369	Mahe	La Reserve	X	X	X	X	X
H. brevis	SM	378	Mahe	La Reserve	X	X	X	X	X
H. brevis	UMMZ	31451	Mahe	Grand Bois River	X	X	X		X
H. cf. *brevis* CR	SM	284	Mahe	Congo Rouge	X	X	X	X	
H. cf. *brevis* CR	SM	285	Mahe	Congo Rouge	X	X	X	X	
H. cf. *brevis* Praslin	SM	282	Praslin	Fond Peper	X	X	X	X	X
H. cf. *brevis* Praslin	SM	295	Praslin	Fond Peper	X	X		X	X
H. cf. *brevis* Praslin	SM	299	Praslin	Fond Peper	X	X	X	X	X
H. cf. *brevis* Praslin	SM	300	Praslin	Fond Peper	X	X	X	X	X
H. cf. *brevis* Praslin	SM	301	Praslin	Fond Peper	X	X	X	X	X

H. cf. *brevis* Praslin	SM	302	Praslin	Fond Peper	X	X	X	X	X
H. cf. *brevis* Praslin	SM	304	Praslin	Fond Peper	X				
H. cf. *brevis* Praslin	SM	305	Praslin	Fond Peper	X				
H. cf. *brevis* Praslin	SM	306	Praslin	Fond Peper	X				
H. cf. *brevis* Praslin	SM	331	Praslin	Above Fond Peper	X	X	X	X	X
H. cf. *brevis* Praslin	SM	332	Praslin	Above Fond Peper	X	X	X	X	X
H. cf. *brevis* Praslin	SM	333	Praslin	Above Fond Peper	X	X	X	X	X
H. cf. *brevis* Praslin	SM	334	Praslin	Above Fond Peper	X	X	X	X	X
H. cf. *brevis* Praslin	SM	490	Praslin	Coco-De-Mer hotel grounds	X	X	X	X	X
H. cf. *brevis* Praslin	SM	491	Praslin	Fond Peper	X				
H. cf. *brevis* Praslin	SM	492	Praslin	Fond Peper	X				
H. rostratus	SM	288	Cerf		X		X	X	X
H. rostratus	SM	289	Cerf		X	X	X	X	X
H. rostratus	UMMZ	31679	Curieuse	MARSH ON SIDE FACING Praslin	X	X	X	X	X
H. rostratus	UMMZ	31680	Curieuse	MARSH ON SIDE FACING Praslin	X	X	X	X	X
H. rostratus	SM	484	Felicite		X	X	X	X	X
H. rostratus	SM	485	Felicite		X	X	X	X	X
H. rostratus	UMMZ	31109	Fregate	Settlement plateau	X	X	X	X	X
H. rostratus	UMMZ	31110	Fregate	Settlement plateau	X		X	X	X
H. rostratus	SM	268	La Digue	Anse Gaulettes	X				
H. rostratus	UMMZ	31171	La Digue	Plateau	X	X	X		X
H. rostratus	UMMZ	31172	La Digue	Plateau	X		X		X
H. rostratus	SM	182	Mahe	Case Dent	X				
H. rostratus	SM	183	Mahe	Case Dent	X				

H. rostratus	SM	358	Mahe	Chemin Dame Le Roi	X	X	X		
H. rostratus	SM	359	Mahe	Chemin Dame Le Roi	X	X	X		X
H. rostratus	SM	363	Mahe	Les Canelles	X	X	X		X
H. rostratus	SM	364	Mahe	Les Canelles	X	X	X	X	X
H. rostratus	UMMZ	31219	Mahe	Mt. Simpson	X	X	X	X	X
H. rostratus	UMMZ	31220	Mahe	Mt. Simpson	X	X	X	X	X
H. rostratus	SM	277	Praslin	Fond Peper	X				
H. rostratus	SM	279	Praslin	Fond Peper	X				
H. rostratus	SM	280	Praslin	Fond Peper	X				
H. rostratus	SM	475	Praslin	Zimbabwe	X		X		
H. rostratus	SM	476	Praslin	Zimbabwe	X	X			
H. rostratus	UMMZ	31660	Praslin	Between Vallee de Mai and Baie Ste Anne	X	X	X	X	X
H. rostratus	UMMZ	31756	Praslin	La Plaine Hollandaise	X	X		X	X
H. rostratus	UMMZ	31562	Ste Anne	SW side of island	X	X	X	X	X
H. rostratus	UMMZ	31571	Ste Anne	SW side of island	X	X	X	X	X
H. rostratus	SM	207	Ste Anne	Jardin Maron	X				
H. rostratus	SM	209	Ste Anne	Jardin Maron	X				
H. rostratus	SM	229	Ste Anne	Anse Mordon trail	X	X	X		X
H. rostratus	SM	230	Ste Anne	Anse Mordon trail	X				
H. rostratus	SM	232	Ste Anne	Anse Mordon trail	X				
H. rostratus	SM	410	Ste Anne	Mare Aux Cochons	X	X	X	X	X
H. rostratus	UMMZ	31409	Ste Anne	La Passe side of Gratte Fess Trail	X	X	X	X	X
H. rostratus	UMMZ	31410	Ste Anne	La Passe side of Gratte Fess Trail	X	X	X	X	X
P. cooperi	SM	469	Mahe	Mare Aux Cochons trail	X				
P. cooperi	UMMZ	31309	Mahe	Mt. Coton	X	X	X	X	X
P. cooperi	UMMZ	31310	Mahe	Mt. Coton	X	X	X	X	X
P. cooperi	UMMZ	31311	Mahe	Grand Bois River	X	X	X		X
P. cooperi	UMMZ	31322	Mahe	Grand Bois River	X	X	X	X	X
P. cooperi	UMMZ	31631	Mahe	Grand St Louis River	X	X	X	X	X
P. cooperi	UMMZ	31861	Mahe	Mt. Simpson	X	X	X	X	X
P. cooperi	SM	237	Silhouette	Jardin Maron	X				
P. cooperi	UMMZ	31305	Silhouette	Grand Barbe	X	X	X	X	X
P. cooperi	UMMZ	31306	Silhouette	Grand Barbe	X		X	X	
P. cooperi	UMMZ	31307	Silhouette	Grand Barbe	X	X	X	X	X

P. cooperi	UMMZ	31308	Silhouette	Grand Barbe	X	X	X	X	X
P. cooperi	UMMZ	31452	Silhouette	Grand Barbe	X	X	X	X	X

APPENDIX 5

Details of samples used in Chapter 6

Details of samples used and sequence data generated in Chapter 6. Museum code SM refers to samples collected during this thesis and RAN refers to samples housed at the University of Michigan Museum of Zoology. An “X” symbol indicates that novel sequences were generated.

Species	Code	Island	*16s*	*cytb*	*rag1*	*pomc*	*bdnf*	*h3*	*slca81*	*rho*	*rost5*	*brev5*	*alt23*	*sech5*	*rost1*	*brev2*	*alt15*
H. cf. *brevis* CR	SM284	Mahé	X	X	X	X	X	X	X	X		X		X		X	X
	SM285	Mahé	X	X	X	X	X	X	X	X		X		X		X	X
H. cf. *brevis* Praslin	SM295	Praslin	X	X	X	X	X	X		X	X		X	X		X	X
	SM299	Praslin	X	X	X	X	X		X	X	X	X	X			X	
	SM331	Praslin		X	X	X	X	X	X	X	X	X	X	X		X	
	SM282	Praslin	X														
	SM304	Praslin	X	X													
	SM300	Praslin	X	X													
	SM301	Praslin	X	X													
	SM302	Praslin	X	X													
	SM305	Praslin	X	X													
	SM306	Praslin	X	X													
	SM331	Praslin	X														
	SM332	Praslin	X	X													
	SM333	Praslin	X	X													
	SM334	Praslin	X	X													
	SM490	Praslin	X	X													
	SM282	Praslin		X													

G. alternans	RAN31051	Fregate	X		X	X	X	X	X	X	X	X	X	X	X	X	
	RAN31062	Fregate	X		X	X	X	X		X	X	X	X	X	X	X	X
	RAN31052	Fregate	X														
	RAN 31053	Fregate	X														
	RAN 31054	Fregate	X														
	RAN 31055	Fregate	X														
	RAN 31056	Fregate	X														
	RAN 31057	Fregate	X														
	RAN 31058	Fregate	X														
	RAN 31059	Fregate	X														
	RAN 31060	Fregate	X														
	RAN 31061	Fregate	X														
	RAN 31063	Fregate	X														
	RAN 31064	Fregate	X														
	RAN 31065	Fregate	X														
	RAN 31066	Fregate	X														
	RAN 31067	Fregate	X														
	RAN 31074	Fregate	X														
	RAN 31235	Silhouette	X														
	RAN 31349	Silhouette	X														
	RAN 31351	Silhouette	X														
	SM318	La Digue			X	X	X		X	X	X		X	X	X	X	X
	RAN31314	Mahé	X		X	X	X	X	X	X	X	X	X	X	X	X	X
	SM159	Mahé	X		X	X	X	X		X	X	X	X	X	X	X	X
	SM130	Praslin	X		X	X	X	X	X	X	X	X	X	X	X	X	X
	RAN31133	Silhouette	X		X	X	X	X		X	X	X	X		X	X	X
	RAN 31134	Silhouette	X														
	RAN 31138	Silhouette	X														
	RAN 31139	Silhouette	X														
	RAN 31236	Silhouette	X														

	RAN 31237	Silhouette	X														
	RAN 31238	Silhouette	X														
	RAN 31243	Silhouette	X														
	RAN 31245	Silhouette	X														
	RAN 31247	Silhouette	X														
	RAN 31249	Silhouette	X														
	RAN 31135	Silhouette	X														
	RAN 31352	Silhouette	X														
	RAN 31353	Silhouette	X														
	RAN 31354	Silhouette	X														
	RAN 31355	Silhouette	X														
	RAN 31356	Silhouette	X														
	RAN 31357	Silhouette	X														
	RAN 31358	Silhouette	X														
	RAN 31395	Silhouette	X														
	SM131	Praslin	X	X													
	SM160	Mahe	X														
	SM161	Mahe	X														
	SM297	Praslin	X	X													
	SM303	Praslin	X	X													
	RAN31242	Silhouette	X		X	X	X	X	X	X	X	X	X	X	X	X	X
	SM318	La Digue	X	X													
	SM185	Mahe		X													
	SM181	Mahe		X													
	SM186	Mahe		X													
	SM323	La Digue		X													
	SM324	La Digue		X													
	SM325	La Digue		X													
	SM329	La Digue		X													
G. larvata	RAN31187	La Digue	X		X	X	X		X	X	X	X	X	X	X	X	X
	RAN31202		**X**		X			X	X	X							

RAN 25168	La Digue	X														
RAN 31188	La Digue	X	X													
RAN 31189	La Digue	X														
RAN 31191	La Digue	X														
RAN 31192	La Digue	X														
RAN 31201	Mahe	X														
RAN 31468	Silhouette	X														
RAN 31472	Silhouette	X														
RAN 31473	Silhouette	X														
RAN 31477	Silhouette	X														
RAN 31598	Ste Anne	X	X													
RAN 31190	La Digue	X														
RAN 31674	La Digue	X														
RAN 31761	Praslin	X														
RAN 31828	La Digue	X														
RAN 31829	La Digue	X														
RAN 31830	La Digue	X														
RAN 31831	La Digue	X														
RAN 31832	La Digue	X														
RAN 31834	La Digue	X	X													
RAN31203	Mahé			X	X	X	X			X	X	X	X	X	X	X
RAN31760	Praslin	X		X	X	X	X	X	X	X	X		X	X	X	X
RAN31364	Silhouette	X	X	X	X	X		X		X	X	X	X	X	X	X
SM132	Praslin	X	X													
SM238	Silhouette	X	X													
SM239	Silhouette	X	X													
SM240	Silhouette	X	X													
SM241	Silhouette	X	X													
SM250	Silhouette	X	X													
SM281	Praslin	X	X													
RAN 31236	Silhouette		X													
RAN 31237	Silhouette		X													

	SM231	Silhouette		X													
G. sechellensis	RAN31312	Mahé	X		X	X		X		X		X	X	X	X	X	
	RAN 25768	Mahe	X														
	RAN 25769	Mahe	X														
	RAN 26802	Mahe	X														
	RAN 26803	Mahe	X														
	RAN 26804	Mahe	X														
	RAN 26886	Mahe	X														
	RAN 26887	Mahe	X														
	RAN 31453	Silhouette	X														
	RAN 31489	Silhouette	X														
	RAN 31490	Silhouette	X														
	RAN 31491	Silhouette	X														
	RAN 31492	Silhouette	X														
	RAN 31494	Silhouette	X														
	RAN 31495	Silhouette	X														
	RAN 31496	Silhouette	X														
	RAN 31497	Silhouette	X														
	RAN 31600	Mahe	X														
	RAN 31602	Mahe	X														
	RAN 31629	Mahe	X														
	RAN 34729	Mahe	X														
	RAN 31486	Silhouette		X													
	RAN31323	Mahé	X		X	X		X	X	X		X	X	X	X	X	X
	RAN31249				X			X	X	X							
	SM164			**X**							X						
	SM278	Praslin	X		X	X	X	X	X	X	X	X	X	X	X	X	X
	RAN 31380	Silhouette	X														
	RAN 31381	Silhouette	X														
	RAN 31382	Silhouette	X														

	RAN 31383	Silhouette	X														
	RAN 31384	Silhouette	X														
	RAN 31385	Silhouette	X														
	RAN 31387	Mahe	X														
	RAN 31389	Mahe	X														
	RAN 31390	Mahe	X														
	RAN 31488	Silhouette	X														
	RAN31379	Silhouette	X		X	X	X	X	X	X		X	X	X	X	X	X
	SM190	Mahe	X	X													
	SM194	Mahe		X													
	SM210	Silhouette		X													
	SM248	Silhouette		X													
	SM249	Silhouette		X													
	SM298	Praslin		X													
	SM327	Praslin		X													
H. brevis	RAN31451	Mahé	X		X		X	X	X	X	X	X	X	X	X	X	X
	SM162	Mahé	X	X		X	X	X	X		X	X					X
	SM163	Mahe	X	X													
	SM192	Mahé	X	X	X	X	X	X		X		X		X	X	X	X
	SM193	Mahé	X	X	X	X		X	X	X	X	X	X	X		X	
	SM283	Mahe	X	X													
	SM354	Mahe	X	X													
	SM355	Mahe	X	X													
	SM356	Mahe	X	X													
	SM366	Mahe	X	X													
	SM367	Mahe	X	X													
	SM368	Mahe	X	X													
	SM369	Mahe	X	X													
	SM378	Mahe	X														
H. rostratus	RAN31109	Fregate	X	X	X	X	X	X		X	X	X	X	X	X	X	X

	31110	Fregate	X	X													
	RAN31219	Mahé	X	X	X	X	X	X		X	X	X	X	X	X	X	X
	RAN31223				X			X	X	X							
	SM358	Mahé	X	X	X		X	X		X		X		X	X	X	X
	RAN 31171	La Digue	X	X													
	RAN 31172	La Digue	X	X													
	RAN 31220	Mahe	X														
	RAN 31410	Silhouette	X														
	RAN 31562	Ste Anne	X	X													
	RAN 31571	Ste Anne	X	X													
	RAN 31679	Curieuse	X	X													
	RAN 31680	Curieuse	X	X													
	RAN 31756	Praslin	X	X													
	RAN 31410	Silhouette		X													
	RAN 31220	Mahe		X													
	RAN31660	Praslin	X	X	X	X	X	X		X	X	X	X	X	X	X	X
	RAN31409	Silhouette	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	SM183	Mahe		X													
	SM207	Silhouette		X													
	SM209	Silhouette		X													
	SM232	Silhouette		X													
	SM268	La Digue		X													
	SM277	Praslin		X													
	SM279	Praslin		X													
	SM280	Praslin		X													
	SM288	Cerf		X													
	SM289	Cerf		X													
P. cooperi	RAN31309	Mahé	X	X	X	X	X	X	X	X	X	X		X	X	X	X
	RAN31308	Silhouette	**X**										X				
	RAN31310	Mahé	X		X	X	X	X	X	X	X	X		X	X		X
	RAN 31307	Silhouette	X														

	RAN 31311	Mahe	X														
	RAN 31322	Mahe	X														
	RAN 31306	Silhouette	X														
	RAN 31452	Silhouette	X														
	RAN 31631	Mahe	X														
	RAN 31861	Mahe	X														
	SM237	Silhouette	X	X													
	RAN31305	Silhouette	X		X	X	X	X	X	X	X	X	X		X		X