POPULATION GENOMIC AND PHENOTYPIC DIVERSIFICATION IN AFRICAN REED FROGS

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POPULATION GENOMIC AND PHENOTYPIC DIVERSIFICATION IN AFRICAN REED FROGS

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On a global scale, taxonomic and phenotypic diversity result from diversification and extinction operating across all levels of biological organization, from populations to species, communities, and biomes. At each of these levels, extrinsic mechanisms like climate are interacting with organismal traits like dispersal ability to shape global patterns of species diversity and to drive phenotypic evolution. This dissertation focuses on how global climate shifts and isolation on oceanic islands drive evolutionary processes and patterns of community assembly and how organismal differences in habitat use and breeding biology influence species responses to these shared global events. Reed frogs (Hyperoliidae) are an ideal group for asking questions about broad-scale patterns of diversification because there are over 200 species broadly distributed throughout sub-saharan Africa in rainforest, bushland and savannah habitats and there are at least two cases of overseas dispersal to oceanic islands. They also exhibit a number of unusual traits including sexual dichromatism, a form of sexual dimorphism where males and females are different colors, a diverse assortment of reproductive modes, and physiological adaptations for living in arid environments, which provides a rich framework for investigating the mechanisms that shape this phenotypic diversification as well as how these phenotypes mediate species'

responses to environmental change. Chapter 1 describes the prevalence of sexual dichromatism in frogs (including Hyperoliidae) and outlines future lines of research for understanding the evolution and function of this unusual trait. Chapter 2 investigates potential dispersal routes for reed frogs that colonized the oceanic islands of São Tomé and Príncipe in the Gulf of Guinea. Chapter 3 uses population genomic approaches to characterize inter-island dispersal and *in situ* speciation in reed frogs endemic to the Gulf of Guinea islands. Finally, Chapter 4 employs a comparative phylogeographic study across three species of reed frogs that inhabit a spectrum of habitats to investigate mechanisms shaping diversification in the Guineo-Congolian forest of Central Africa and the land-bridge island Bioko.

BIOGRAPHICAL SKETCH

Rayna Camille Bell was raised by Mark and Elisabeth in Fairfax, California, an idyllic small town nestled in the redwoods and a short drive from the Point Reyes seashore. Rayna attended the College of Marin for two years where her interests in organismal biology developed thanks to her biology professor Joe Mueller and a summer field ecology course in Alaska. In 2005, Rayna transferred to the University of California, Berkeley and began an undergraduate research apprenticeship at the Museum of Vertebrate Zoology with Jason MacKenzie and Craig Moritz. Over the subsequent two years, Rayna reconstructed patterns of molecular variation in rainforest-restricted frogs and lizards endemic to the Australian Wet Tropics to understand how spatial population histories correlate with historic climate fluctuations. The museum became her home base on campus, and interactions with enthusiastic faculty, postdoctoral researchers, and graduate students ultimately shaped her interest in pursuing a graduate degree in evolutionary biology. In 2008 Rayna joined the Zamudio Lab in Ecology & Evolutionary Biology at Cornell University. During her first year she completed laboratory rotations as a Presidential Life Sciences Fellow where she worked on population genetic structure in a Brazilian Atlantic Forest frog with Kelly Zamudio, genetic bottlenecks in African village dogs with Carlos Bustamante, and mating system biology in Hawaiian crickets with Kerry Shaw. For her dissertation research, Rayna focused on micro-evolutionary processes that shape genetic differentiation in Central African reed frogs. This research took her to the rainforests of Gabon, the land-bridge island of Bioko in Equatorial Guinea, and the oceanic

islands of São Tomé and Príncipe. During her first trip to Gabon in 2009, Rayna noticed that several of her focal species exhibit sexual dichromatism, a form of sexual dimorphism in which males and females are different colors. This observation led to a review paper (Chapter 1) documenting the prevalence of sexual dichromatism in frogs and outlining future lines of research to understand the evolution and function of this trait in frogs. Rayna plans to address some of these hypotheses as a Chancellor's Postdoctoral Fellow with Jimmy McGuire in the Museum of Vertebrate Zoology at the University of California, Berkeley.

To the Museum of Vertebrate Zoology at the University of California, Berkeley,
for cultivating my passion and confidence to pursue organismal research.

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I am tremendously grateful to the faculty in the Department of Ecology & Evolutionary Biology at Cornell for contributing to my development as a scientist and teacher over the past six years. In particular I would like to thank Amy McCune for expanding my evolutionary perspective to include macro-evolutionary timescales, Paul Feeney for enthusiastically supporting my initial dissertation project ideas, Monica Geber for constructive feedback on my doctoral dissertation improvement grant, and Andre Kessler for taking a foray into frog skin pigment composition. I am also grateful to the numerous faculty in other departments on campus that kindly shared their time and expertise with me, including Ellis Lowe for teaching me how to quantify spectral sensitivity in frog retinas, Carl Hopkins for guidance on how to plan my first field season in Gabon, and Elizabeth Adkins-Regan for generously sharing

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be a better scientist.

Over the course of my Ph.D. I spent a substantial amount of time in the field and I am grateful to my main collaborators Bryan Stuart, Patrick MacLaughlin, and Robert Drewes for sharing the highs and lows of fieldwork with me. Fieldwork in Gabon would not have been possible without logistical support from the Wildlife Conservation Society, the Agence National des Parcs Nationaux, the Organisation Ecotouristique du Lac Oguemoué, and assistance in the field from Nicolas Emba-Yao, Faustin Moiniyoko, Bryan Hylayre, Eddy Ekomy, Antoine Dibata, Toussaint Ogombet, Ulrich Eyagui, Prosper Endazokou, Pascal Minko, Toussaint Essone, Heather Arrowood, and Cyrille Mvele. I am especially grateful to Dr. Flore Koumba and Madame Keita for helping me secure research and export permits on short notice. I thank Gail Hearn, the Bioko Biodiversity Protection Program, and Universidad Nacional de Guinea Ecuatorial for supporting fieldwork on Bioko, and Andrew Fertig, Djbrilla Ousmane, David Montgomery, Barrett Miles, and Daniel Matute for assistance in the field. For fieldwork in São Tomé and Príncipe, I thank the Ministry of Environment for permission to collect and export specimens, STeP Up São Tomé (Ned Seligman, Roberta dos Santos, and Quintino Quade Cabral) and Bom Bom resort and the Omali Lodge for logistical support, and Jens Vindum, Andrew Stanbridge, João Pedro Pio, Brian Simison, Velma Schnolll, and Roberta Ayres for assistance in the field. A special thank you to Chris Dalton for driving me to JFK in a blizzard to catch my flight to Bioko and for tracking down my lost bag en route to Gabon. Thank you to Charles Dardia, John Friel and the Cornell University Museum of Vertebrates

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CHAPTER 1

SEXUAL DICHROMATISM IN FROGS: NATURAL SELECTION, SEXUAL SELECTION, AND UNEXPECTED DIVERSITY[1]

Abstract

Sexual dichromatism, a form of sexual dimorphism in which males and females differ in color, is widespread in animals but has been predominantly studied in birds, fishes and butterflies. Moreover, although there are several proposed evolutionary mechanisms for sexual dichromatism in vertebrates, few studies have examined this phenomenon outside the context of sexual selection. Here, we describe unexpectedly high diversity of sexual dichromatism in frogs and create a comparative framework to guide future analyses of the evolution of these sexual color differences. We review what is known about evolution of color dimorphism in frogs, highlight alternative mechanisms that may contribute to the evolution of sexual color differences, and compare them to mechanisms active in other major groups of vertebrates. In frogs, sexual dichromatism can be *dynamic* (temporary color change in males) or *ontogenetic* (permanent color change in males or females). The degree and duration of sexual color differences vary greatly across lineages, and we do not detect phylogenetic signal in the distribution of this trait, therefore frogs provide an opportunity to investigate the roles of natural and sexual selection across multiple independent derivations of sexual dichromatism.

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1.1 Introduction

Sexual dichromatism, a form of sexual dimorphism in which males and females differ in *color*, is widespread in animals and is most commonly studied in birds (Kimball & Ligon, 1999, Badyaev & Hill 2003) fishes (Kodric-Brown 1998), and butterflies (Allen *et al.* 1998). In *The Descent of Man* (1874), Darwin highlighted the strong association between sexually dimorphic traits and related courtship behaviors, thus setting the stage for sexual selection as a primary evolutionary mechanism for sexual dimorphism. In frogs and toads (anurans), the most common form of sexual dimorphism is body size (more than 90% of species), and these differences are attributed to fecundity (when females are larger; Salthe & Duellman 1973) or sexual selection (when males are larger; Shine 1979). Prior to this study, sexual dichromatism was only known from 25 species (or less than 0.5%) of frogs (Hoffman & Blouin 2000). Though we have now documented sexual dichromatism in over 120 species (see Table 1.S1), both its function and evolution remain poorly understood. In this review, we (1) document the distribution and diversity of sexual dichromatism in frogs, (2) test whether the phylogenetic distribution of sexual dichromatism reflects shared evolutionary history, (3) identify circumstances in which sexual selection versus other selective mechanisms may be involved in maintaining sexual dichromatism, and (4) outline areas of future research related to the evolution and function of sexual dichromatism in frogs.

1.2 The diversity of sexual dichromatism in frogs

Within frogs, we make a distinction between two broad classes of sexual dichromatism. In the first class, which we refer to as *dynamic dichromatism*, males undergo a temporary color change during the breeding season (Figure 1.1a-b). The duration of this dynamic color change varies across species from only a few hours (e.g *Incilius luetkenii;* Doucet & Mennill 2010) to several days or weeks during the breeding season (e.g. *Rana temporaria;* Hedengren 1987). In the second class of dichromatism, which we refer to as *ontogenetic dichromatism*, either males or females undergo a permanent color and/or color pattern change, generally at the onset of sexual maturation (Figure 1.1c-d). The degree of color differentiation between the sexes ranges from subtle differences in shade (e.g. *Scaphiophryne gottlebei;* Glaw & Vences 1994) to dramatic differences in both color and pattern (e.g. *Hyperolius argus;* Stewart 1967).

We distinguish between the two classes of dichromatism (dynamic and ontogenetic), and their respective phylogenetic distributions, because they may have important differences in terms of evolutionary lability and function. Dynamic sexual dichromatism is present in 31 species from nine families and subfamilies (Table 1.S1) and is especially prevalent in the Ranidae, Bufonidae and Hylidae (Figure 1.2a). Due to its ephemeral nature, this class of dichromatism is likely under-documented in the literature and may be far more common among frogs. In particular, we anticipate future records of dynamic dichromatism within lineages where it has already been documented and is fairly common (e.g. Bufonidae). Ontogenetic dichromatism appears to be more taxonomically widespread and is present in 92 species from 18

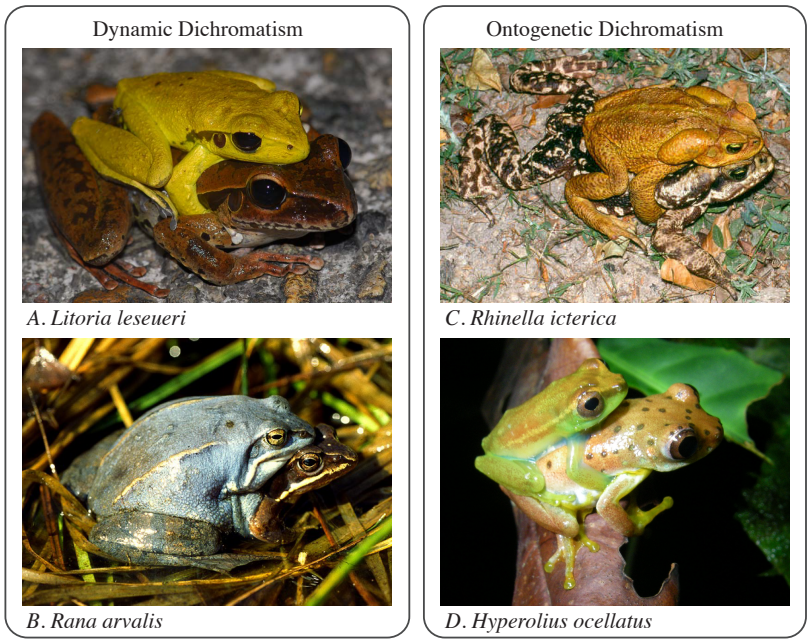
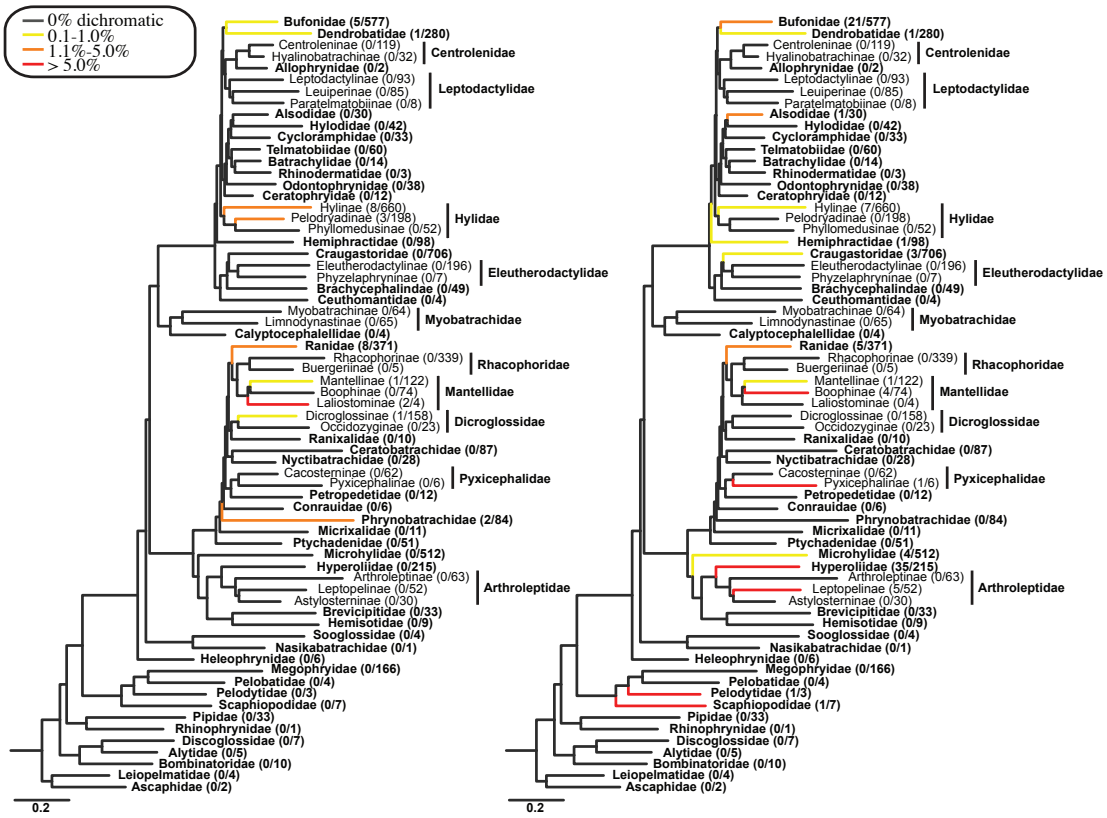


Figure 1.1: Examples of frog species showing dynamic sexual dichromatism (A, B), and ontogenetic dichromatism (C, D). (A) *Litoria leseueri* (Hylidae): males turn yellow for several days during the breeding season (Photo credit: Stewart Macdonald); (B) *Rana arvalis* (Ranidae): males turn blue for several weeks during the breeding season (Photo credit: Lars Iversen); (C) *Rhinella icterica* (Bufonidae): at sexual maturity males are yellow and females are mottled brown and tan. Females retain the juvenile coloration (Photo credit: Célio F. B. Haddad). (D) *Hyperolius ocellatus* (Hyperoliidae): at sexual maturity males are green with white dorsolateral lines and females are rusty red to silver with small spots. Males retain the juvenile coloration (Photo credit: Rayna C. Bell).



A. Dynamic Dichromatism B. Ontogenetic Dichromatism

Figure 1.2: Phylogenetic distribution of dynamic sexual dichromatism (A) and ontogenetic sexual dichromatism (B). Families are shown in bold and subfamilies in regular print. Branches are colored according to the percentage of dichromatic species in each clade and the proportion of dichromatic species is shown in parentheses for each tip. The phylogeny is modified from Pyron & Wiens 2011.

families and subfamilies (Table 1.S1), though the vast majority of these dichromatic species are in the Hyperoliidae, Bufonidae and Hylidae (Figure 1.2b).

Dynamic dichromatism is only present in the "neobatrachia", or modern lineages, of frogs whereas ontogenetic dichromatism is present in several basal lineages as well as the three major modern lineages. These differences in phylogenetic distribution may provide insight into the underlying physiological mechanisms for each type of color change and whether similar pathways are employed in both types of dichromatism and across multiple independent evolutionary origins. The species-rich lineages in which sexual dichromatism is absent may be equally informative for understanding the evolution and genetic basis of this trait. For instance, dynamic sexual dichromatism is entirely absent from the primarily ground-dwelling Microhylidae, in which a heavy reliance on crypsis in leaf litter may render temporary male color change too costly. Alternatively, dichromatism may be absent in these lineages due to developmental constraint.

1.3 Characterizing the phylogenetic distribution of sexual dichromatism in frogs

To test for phylogenetic signal in each class of sexual dichromatism we used the most comprehensive amphibian phylogeny to date (Pyron & Wiens 2011), which includes representatives from more than 90% of the currently recognized genera and approximately 2,400 species (nearly 40% of total frog species diversity). We pruned

the Pyron & Wiens tree (2011) to the family or sub-family level as applicable, and created an ultrametric version of this tree using the function *chronopl*, with lambda=0 to approximate non-parametric rate smoothing (Sanderson 2002). Character states for dynamic and ontogenetic dichromatism were then assigned to the appropriate tips (families or sub-families).

Phylogenetic signal is a measure of how well shared evolutionary history explains the distribution of trait values among terminal taxa and a particular phylogeny. We quantified the degree of phylogenetic signal in both classes of sexual dichromatism using Pagel's lambda (λ; Pagel 1999), a test statistic that varies from zero to one, where a value of zero indicates that trait evolution is independent of phylogeny and a value of one indicates that shared characters states among terminal taxa reflect shared ancestry. We optimized the value of lambda for both classes of sexual dichromatism using maximum likelihood in the *fitDiscrete* function of geiger with an equal rates character state transition model (Harmon *et al.* 2009). To determine whether our phylogenetic signal estimates were significantly greater than zero, we compared the negative log likelihood values for our original phylogeny with those obtained after transforming the branches in the phylogeny by lambda=0 using the *lambdaTree* function of Geiger (Harmon *et al.* 2009), which results in a phylogeny without phylogenetic signal. All analyses were performed in R version 2.13.1.

Although our current numbers of sexually dichromatic frogs are likely underestimated, this review significantly improves our current understanding of the phylogenetic

distribution and diversity of this trait. Both ontogenetic dichromatism ($\lambda_{original}$ = 0.000045, log likelihood = -37.52307; $\lambda_{transformed}$ = 0, log likelihood = -37.52303) and dynamic dichromatism ($\lambda_{original}$ = 0.000045, log likelihood = -27.12687; $\lambda_{transformed}$ = 0, log likelihood = -27.12684) exhibit values of phylogenetic signal that are not significantly different from zero, indicating that trait evolution is independent of phylogeny. These values indicate that history alone cannot explain the phylogenetic distribution of either dynamic or ontogenetic dichromatism in frogs. Broad macroevolutionary patterns, however, point to specific lineages that merit further study and direct our attention to a diversity of evolutionary mechanisms that may result in sexual dichromatism.

1.4 Sexual dichromatism and sexual selection

In vertebrates, sexual dichromatism can exists in three general classes: i) brightly colored males and drab females, ii) brightly colored females and drab males, and iii) both sexes equally conspicuous but with differences in color and/or color pattern. Regardless of the particular class of dichromatism, most studies of sexually dichromatic vertebrates find support for sexual selection as a driving force in the origin and maintenance of this trait. For instance, when males are the brighter sex, male-male competition and female choice are cited as evolutionary mechanisms in a number of vertebrate taxa including birds (Andersson 1982), fishes (Kodric-Brown & Brown 1984), lizards (Olsson 1992, Wiens *et al.* 1999), turtles (Moll *et al.* 1981), salamanders (Salthe 1967, Todd & Davis 2007), and primates (Cooper & Hosey 2003,

Caro 2009). In cases where females are brighter than males, sexual color differences may be explained by a sex-role reversal in mating system in which females compete for males (Andersson 1994). Finally, when both sexes are bright and differ in coloration, these differences are often attributed to mutual-mate choice, where males and females evaluate the quality of potential mates based on coloration (Hanssen *et al.* 2006).

While sexual selection may be the prevailing evolutionary mechanism underlying sexual dichromatism in vertebrates, alternative mechanisms need to be considered. Sexual niche partitioning, in which males and females use different resources or experience different predation pressures, is implicated in a number of sexually dimorphic taxa (Feduccia & Slaughter 1973, Partridge & Green 1985, Shine 1989). Relative to sexual selection, this theory remains largely unexplored in the scientific literature, particularly in the context of sexual dichromatism (but see Heinsohn *et al.* 2005). The historical bias towards sexual selection may be inherent to the groups that have traditionally been studied, such as birds and fishes, that typically have polygynous or promiscuous mating systems with highly visual courtship displays for mate selection. Though dynamic dichromatism in diurnal frogs may be consistent with sexual selection (e.g. Doucet & Mennill 2010), ontogenetic dichromatism in nocturnal species where females and males are equally conspicuous indicates that ecological selection may also be an important selective force. Therefore, sexual dichromatism in frogs, and in particular ontogenetic dichromatism, provides the opportunity to

investigate the relative roles of natural and sexual selection across multiple independent derivations of this trait.

1.5 Evolutionary mechanisms for dynamic sexual dichromatism

Dynamic sexual dichromatism in frogs is likely driven by sexual selection because these temporary color changes only occur in males and coincide with the mating season (Doucet & Mennill 2010, Hettyey *et al*. 2009). Within the realm of sexual selection this class of dichromatism may serve a variety of functions that are well characterized in other taxa. These potential functions include male-male competition, which is well documented in birds (Andersson 1994), visual signaling between the sexes, which is recognized in at least one frog species (Taylor *et al*. 2007), and as an honest indicator of mate quality, which has been proposed in birds (Hamilton & Zuk 1982), lizards (Martín & López 2010) and some frogs (Vásquez & Pfennig 2007). Though these functions are well characterized in other vertebrate groups, the specific functions of dynamic dichromatism may vary greatly across frog lineages depending on certain aspects of mating system biology, such as reproductive mode and degree of parental care. For instance, in birds, male coloration is a common signal of male quality including paternal investment in offspring and male genotypic quality (Hill 1991), whereas in frogs, females typically assess male quality based on body size and advertisement call (e.g. Pfennig & Tinsley 2002). Nonetheless, females may use carotenoid-based color as an honest indicator of male quality in breeding aggregations where acoustic signals are more difficult to assess (Vásquez & Pfennig 2007).

Likewise, the duration of temporary color change across dichromatic species likely varies with mating aggregation size and duration of the breeding season. Migratory birds are 23 times more likely to be dichromatic than non-migratory species, which is hypothesized to be due to a shorter mate-sampling period for migratory species (Badyaev & Hill 2003, Friedman *et al*. 2009). Therefore, if male coloration in frogs is in fact used to evaluate mate quality we expect that dynamic dichromatism will be more common in "explosive breeders," or species with shorter breeding seasons (Wells 1977); however, the data to test this hypothesis are not yet available.

The underlying physiology of temporary changes in skin coloration and the range of anuran visual acuity may limit the diversity of temporary coloration observed in male frogs. One of the most dramatic temporary color changes in frogs occurs in *Rana arvalis* where males are bright blue for several weeks. This color change may result from destruction of yellow pigments in xanthophores such that blue wavelengths reflected by the iridophores are unfiltered (Box 1.1). In most dynamically dichromatic species, however, temporary coloration in males is either yellower or slightly darker or lighter than the non-breeding coloration. These temporary color changes are likely accomplished by modulating pigment distribution in xanthophores or melanophores (Bagnara 1998). Though there may be physiological limits as to which temporary color changes are possible, the high prevalence of yellow or "brighter" color changes (23 of the 31 dynamic species; Figure 1.3) may provide some insight

BOX 1.1: *Color variation from three pigment cell types in frog skin*

Interactions between three pigment cell types in the dermis underlie both permanent and temporary coloration in frogs. The layer of pigment and light-reflecting cells (the dermal chromatophore unit) in frog skin includes melanophores, which contain melanin, non-reflecting chromatophores called xanthophores or erythrophores, and reflecting chromatophores called iridophores. The upper layer of this dermal chromatophore unit is composed of non-reflecting chromatophores that are called xanthophores when they bestow yellow coloration and erythrophores when they bestow red coloration. The pigments found in these cells include pteridines, which can be synthesized by the chromatophores, or carotenoids, which are metabolized from the diet. The second cell type, the iridophore, is located below the non-reflecting chromatophores and reflects light with platelets of purine "pigments". This layer creates iridescence by diffracting light within the platelets and interacts with the overlying non-reflecting chromatophores to produce bright colors, such as the bright green coloration present in many frogs (Lyerla & Jameson 1968). In the absence of non-reflecting chromatophores, iridophores may bestow a structural blue color (Bagnara 1998). Likewise, when iridophores are reduced, non-reflecting chromatophores may impart bright red and yellow coloration (Frost & Robinson 1984). The third cell type, the melanophore, is the basal-most chromatophore and contains eumelanin that appears black or dark brown. These three layers interact to produce general skin lightening and darkening in response to physiological change (Frost-Mason *et al.* 1994). Short-duration color changes result from hormonal stimulation (primarily Melanocyte Stimulating Hormone and steroid hormones) that causes dispersion or aggregation of pigment-containing organelles (Bagnara 1976). In contrast, permanent or semi-permanent color changes may involve the synthesis or destruction of pigments (Baker 1951).

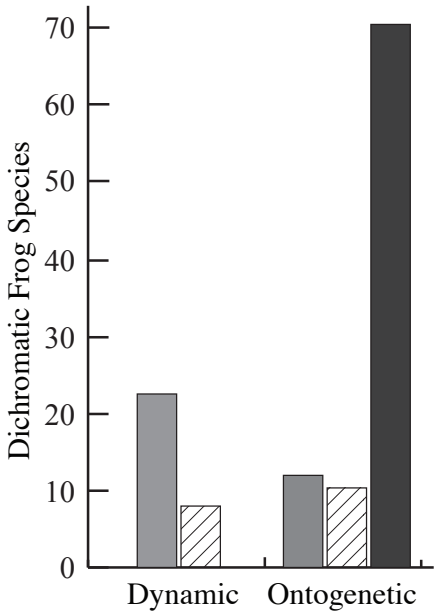


Figure 1.3: Of the frogs that exhibit dynamic sexual dichromatism, males undergo a temporary color change to become yellower or brighter than females in 75% of species (grey bar), while in the remaining 25% of species, males become bluish or darker than females (hashed bar). Of the frogs that exhibit ontogenetic dichromatism, males are more conspicuously colored than females in 13% of species (grey bar), females are more conspicuously colored than males in 11% of species (hashed bar) and males and females are different colored but equally conspicuous in 76% of species (black bar).

into anuran vision, female sensory bias, or developmental constraint in the types of temporary color changes that are possible in frogs.

1.6 Evolutionary mechanisms for ontogenetic sexual dichromatism

Ontogenetic dichromatism, where one sex undergoes a color change that is generally coincident with sexual maturation, may potentially result from a combination of both sexual and natural selection (e.g. Heinsohn *et al*. 2005). The first sub-class of ontogenetic dichromatism, where males are more brightly colored than females, is documented in > 10 frog species (Figure 1.3), the majority of which are found in the Bufonidae and Hylinae. This sub-class of ontogenetic dichromatism is likely subject to similar types of sexual selection as dynamic dichromatism with the exception that sexual color differences are maintained beyond the mating season. Therefore, the relative contribution of sexual selection versus natural selection in these species will presumably depend on the strength of selection for bright and conspicuous coloration during the breeding season and the strength of selection for (or against) that same coloration during non-breeding periods. For chemically-defended frogs, bright coloration in males serves a dual purpose to attract females and as aposematic signals to potential predators (e.g. Bufonidae and Dedrobatidae); therefore, both sexual and natural selection may act in concert in these species to produce brighter coloration in males (Mann & Cummings 2009).

The second sub-class of ontogenetic sexual dichromatism, in which females are equally as conspicuous or more conspicuously colored than males, is especially common in the African hyperoliid treefrogs (35 of the 80 species in this sub-class; Figure 1.3) among which dichromatic species repeatedly evolve from monochromatic species (Veith *et al*. 2009). In several species in this family, sex steroids at the onset of maturation trigger a change in dorsal coloration (Hayes 1997) that results in either bright sexual monochromatism (both sexes become bright at maturity) or sexual dichromatism (females undergo a color change and males retain the juvenile coloration). There are few hypotheses as to the function of color differences in frogs with female-biased ontogenetic dichromatism (Hoffmann & Blouin 2000). Bright coloration in females may be sexually selected, providing a benefit in mutual mate choice (e.g. Hanssen *et al*. 2006) and female-female competition for limited resources or territoriality (e.g. Murphy *et al*. 2009). Alternatively, males and females may utilize different habitats and differences in coloration may simply provide better camouflage in their respective habitats (e.g. sexual niche partitioning; Shine 1989, Heinsohn *et al*. 2005).

Though sexual niche partitioning has never been formally tested as a mechanism for sexual dichromatism in frogs, sexual differences in habitat use have been examined in other dichromatic vertebrates. For instance, in the *Eclectus* parrot, where females are bright red/purple and males are emerald green, both intra-sexual competition and inter-sexual differences in exposure to visual predators contribute to sexual dichromatism (Heinsohn *et al*. 2005). Likewise, in many Old World vipers bright

striped coloration in males, which confuses visual predators (Jackson *et al*. 1976, Pough 1976), is thought to result from increased male exposure to predators when they actively seek females during the breeding season (Shine & Madsen 1994, Lindell & Forsman 1996). Quantifying sexual ecological differences in habitat use and diet (if color differences are carotenoid-based) across multiple lineages of sexually dichromatic taxa may indicate that sexual niche partitioning is a more pervasive mechanism than currently appreciated.

1.7 Future directions and conclusions

Broad ecological factors, such as latitude and range size, correlate with the global distribution of sexual dichromatism in other vertebrates (Badyaev & Hill 2003, Friedman *et al*. 2009) and these macroecological patterns point to specific mechanisms driving the distribution of sexual dichromatism; some of these mechanisms may also be relevant in frogs. For example, birds exhibit higher prevalence of dichromatism in temperate regions, and this pattern may result from increased predation pressure at high latitudes (Martin 1996) resulting in reduced coloration in females (Badyaev & Hill 2003). Conversely, sexual dichromatism in frogs appears to be more common among tropical than temperate species (108 and 15 species, respectively, Table 1.S1). Frogs are ancestrally temperate, and the extensive species diversity in the tropics is driven by diversification in a few, more derived lineages (Wiens 2007); therefore, accounting for the historical effects of latitude on diversification will be necessary to identify whether differences in predation pressure

between temperate and tropical environments affect the global distribution of sexual dichromatism in frogs.

Species range size and species richness of a particular breeding community may also be important predictors of sexual dichromatism in vertebrates. Sexually dichromatic birds tend to have broader distributions than monochromatic species (Badyaev & Ghalambor 1998, Price 1998) and sexual dichromatism is often lost on islands (Peterson 1996). One potential explanation is that selection for dichromatism is correlated with increasing importance of species recognition (Moll *et al*. 1981, Figuerola & Green 2000). If sexual dichromatism in frogs enhances species recognition, we might expect that sexual dichromatism is more common in frog communities that form diverse breeding assemblages where other mating signals, such as call, may be insufficient for correctly identifying conspecifics (Hebets & Papaj 2005).

Finally, ontogenetic dichromatism may also be non-adaptive, particularly in species with distinct juvenile and adult color phases, such as in hyperoliid treefrogs. The ontogenetic color change in these species can result in sexual monochromatism if both sexes undergo an identical color change at sexual maturity, or sexual dichromatism if the ontogenetic pathway is disrupted in one sex such that it retains the juvenile coloration. Because steroid hormones have a similar effect on chromatophores as melanocyte stimulating hormone (Box 1), a change in chromatophore sensitivity to either male or female sex hormones could result in the loss of ontogenetic color

change in only one sex. Characterizing the underlying genetics of ontogenetic color change pathways will be essential for assessing whether non-adaptive evolution can explain the multiple losses of ontogenetic monochromatism, and therefore sexual dichromatism, in this group that accounts for 29% of sexually dichromatic frogs.

Developmental and hormonal skin color regulation is well characterized in several frog species (Bagnara 1960, Barnara *et al*. 1978, Frost 1984), providing an excellent framework for studies of the underlying physiology of dynamic and ontogenetic sexual dichromatism (Box 1). Likewise, the capacity to discern color differences is well documented for several diurnal frog species (Hailman & Jaeger 1974, Kondrashev *et al*. 1976, Siddiqi *et al*. 2004) therefore applying appropriate vision models to studies of sexual selection in diurnal frog species should be feasible. The extent of anuran spectral sensitivity in low light conditions, however, is largely unknown (but see Gomez *et al*. 2010) and will be a necessary component of dichromatism research in nocturnal species.

Our review highlights that we are rapidly gathering data on the distribution of sexual dichromatism among frog species, but that we still know very little about the function of sexual dichromatism in this group of vertebrates. Our review also underscores the potential benefits of using frogs for investigating the relative roles of natural selection and sexual selection in the evolution of sexual dichromatism, and the opportunity for interpreting those patterns in a comparative framework. In particular, studies that focus on lineages in which dynamic or ontogenetic dichromatism evolve repeatedly

hold the most promise for addressing hypotheses about the origin and maintenance of this phenotype in frogs as well as other groups of dichromatic organisms.

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SUPPLEMENTARY INFORMATION FOR CHAPTER 1 - SEXUAL DICHROMATISM IN FROGS: NATURAL SELECTION, SEXUAL SELECTION, AND UNEXPECTED DIVERSITY

Table 1.S1 Species that display dynamic and ontogenetic dichromatism.

Species	Dichromatism	Reference
Aglyptodactylus madagascariensis	Dynamic	Glaw & Vences 2007
Aglyptodactylus securifer	Dynamic	Glaw & Vences 2007
Amietophrynus kassasii	Ontogenetic	Baha El Din 2006
Amietophrynus kisoloensis	Dynamic	Channing 2001
Amietophrynus xeros	Ontogenetic	Rödel 2000
Anaxyrus americanus	Ontogenetic	Wright & Wright 1949
Anaxyrus boreas	Ontogenetic	Elliot *et al*. 2009
Anaxyrus canorus	Ontogenetic	Karlstrom 1973
Atelopus chiriquiensis	Ontogenetic	Savage 2002
Atelopus senex	Ontogenetic	Savage 2002
Atelopus varius	Ontogenetic	Savage 1972
Blommersia angolafa	Ontogenetic	Andreone *et al*. 2010
Boophis albilabris	Ontogenetic	Glaw & Vences 2007
Boophis laurenti	Ontogenetic	Glaw & Vences 2007
Boophis microtympanum	Ontogenetic	Glaw & Vences 1994
Boophis pauliani	Ontogenetic	Glaw & Vences 1994
Bufo bufo	Dynamic	Kuzmin 1999
Bufo japonicus	Dynamic	Maeda & Matsui 1990
Clinotarsus curtipes	Dynamic	B. Das (pers comm)
Cryptothylax greshoffi	Ontogenetic	Schiotz 1999
Dendropsophus bokermanni	Ontogenetic	Riviero 1969
Dendropsophus leucophyllatus	Ontogenetic	Riviero 1969
Dendropsophus minutus	Dynamic	A. Lima (pers comm)
Dendropsophus parviceps	Ontogenetic	Duellman & Trueb 1986
Dyscophus antongilii	Ontogenetic	Glaw & Vences 2007
Dyscophus guineti	Ontogenetic	Glaw & Vences 2007
Gastrotheca andaquiensis	Ontogenetic	Hoffman & Blouin 2000
Guibemantis liber	Dynamic	Glaw & Vences 2007
Heterixalus alboguttatus	Ontogenetic	Schiotz 1999
Heterixalus tricolor	Ontogenetic	Schiotz 1999
Heterixalus variabilis	Ontogenetic	Glaw & Vences 1994
Hoplobatrachus tigerinus	Dynamic	Glaw & Vences 2007
Hylarana aurata	Dynamic	Gunther 2004
Hylarana grisea	Dynamic	S. Richards (pers comm)
Hylarana volkerjane	Dynamic	Gunther 2004
Hylorina sylvatica	Ontogenetic	Barrio 1967
Hyperolius argus	Ontogenetic	Stewart 1967
Hyperolius bocagei (kachalolae)	Ontogenetic	Schiotz 1999
Hyperolius castaneus	Ontogenetic	Laurent 1950
Hyperolius cinnamomeoventris	Ontogenetic	Laurent 1950
Hyperolius concolor	Ontogenetic	Schiotz 1999
Hyperolius discodactylus	Ontogenetic	Laurent 1950
Hyperolius fusciventris	Ontogenetic	Schiotz 1999

Species	Dichromatism	Reference
Hyperolius glandicolor	Ontogenetic	Schiotz 1971
Hyperolius guttulatus	Ontogenetic	Schiotz 1999
Hyperolius kivuensis	Ontogenetic	Laurent 1950
Hyperolius lateralis	Ontogenetic	Schiotz 1999
Hyperolius mariae	Ontogenetic	Schiotz 1971
Hyperolius marmoratus	Ontogenetic	Stewart 1967
Hyperolius nasutus	Ontogenetic	Laurent 1950
Hyperolius ocellatus	Ontogenetic	Laurent 1950
Hyperolius pardalis	Ontogenetic	Schiotz 1999
Hyperolius parkeri	Ontogenetic	Schiotz 1999
Hyperolius phantasticus	Ontogenetic	Schiotz 1999
Hyperolius pickersgilli	Ontogenetic	Channing 2001
Hyperolius picturatus	Ontogenetic	Schiotz 1999
Hyperolius platyceps	Ontogenetic	Schiotz 1999
Hyperolius quinquevittatus	Ontogenetic	Laurent 1957
Hyperolius riggenbachi	Ontogenetic	Schiotz 1999
Hyperolius spatzi	Ontogenetic	Schiotz 1971
Hyperolius tuberculatus	Ontogenetic	Schiotz 1971
Hyperolius tuberilinguis	Ontogenetic	Stewart 1967
Hyperolius viridiflavus karissimbiensis	Ontogenetic	Laurent 1950
Hyperolius viridiflavus viridiflavus	Ontogenetic	Laurent 1950
Hyperolius wermuthi	Ontogenetic	Schiotz 1999
Hyperolius zonatus	Ontogenetic	Schiotz 1999
Hypsiboas boans	Ontogenetic	A. Lima (pers comm)
Hypsiboas fasciatus	Dynamic	A. Lima (pers comm)
Hypsiboas multifasciatus	Dynamic	A. Lima (pers comm)
Incilius luetkenii	Dynamic	Doucet & Mennill 2010
Incilius marmoreus	Ontogenetic	Duellman & Trueb 1986
Incilius melanochlorus	Ontogenetic	Savage 2002
Incilius periglenes	Ontogenetic	Savage 1966
Isthmohyla calypsa	Ontogenetic	Savage 2002
Isthmohyla pseudopuma	Dynamic	Savage 2002
Leptopelis concolor	Ontogenetic	Passmore & Carruthers 1979
Leptopelis flavomaculatus	Ontogenetic	Harper *et al*. 2010
Leptopelis notatus	Ontogenetic	Schiotz 1999
Leptopelis parkeri	Ontogenetic	Schiotz 1999
Leptopelis vermiculatus	Ontogenetic	Schiotz 1999
Litoria jungguy	Dynamic	Tyler & Knight 2009
Litoria lesueuri	Dynamic	Tyler & Knight 2009
Litoria wilcoxii	Dynamic	Tyler & Knight 2009
Mannophryne trinitatis	Dynamic	Duellman & Trueb 1986
Mertensophryne taitana	Ontogenetic	Stewart 1967
Nectophrynoides tornieri	Ontogenetic	Channing & Howell 2006
Oophaga pumilio	Ontogenetic	Mann & Cummings 2009
Osteocephalus leprieurii	Dynamic	Sztatecsny *et al*. 2010
Pedostibes hosii	Ontogenetic	Inger & Stuebing 1997
Pelodytes caucasicus	Ontogenetic	Kuzmin 1999
Peltophryne lemur	Ontogenetic	Matos-Torres 2006
Phrynobatrachus latifrons	Dynamic	Rödel 2000
Phrynobatrachus natalensis	Dynamic	Rödel 2000
Pristimantis bicolor	Ontogenetic	Rueda & Lynch 1983
Pristimantis erythropleura	Ontogenetic	Lynch 1992
Pristimantis factiosus	Ontogenetic	Lynch & Rueda 1998
Pseudepidalea sicula	Ontogenetic	Stöck *et al*. 2008

Species	Dichromatism	Reference
Pseudepidalea viridis	Dynamic	Arnold 2002
Pyxicephalus edulis	Ontogenetic	Rödel 2000
Ramanella variegata	Ontogenetic	Dutta & Manamendra-Arachchi 1996
Rana arvalis	Dynamic	Arnold 2002
Rana graeca	Dynamic	Valakos *et al*. 2008
Rana hosii	Ontogenetic	Manthey & Grossman 1997
Rana longicrus	Ontogenetic	Lue 1990
Rana septentrionalis	Ontogenetic	Kramek & Stewart 1980
Rana sylvatica	Dynamic	Harding 1997
Rana temporaria	Dynamic	Arnold 2002
Rana vaillanti	Ontogenetic	Ramirez *et al*. 1998
Raorchestes chromasynchysi	Ontogenetic	Vijayakumar pers comm
Rhinella icterica	Ontogenetic	Haddad *et al*. 2008
Rhinella marina	Ontogenetic	Easteal 1963
Rhinella veredas	Ontogenetic	Brandão *et al*. 2007
Rhinella yanachaga	Ontogenetic	Lehr *et al*. 2007
Sanguirana aurantipunctata	Ontogenetic	Fuiten *et al*. 2011
Scaphiophryne gottlebei	Ontogenetic	Glaw & Vences 1994
Scaphiopus couchii	Ontogenetic	Stebbins 2003
Scinax fuscovarius	Dynamic	C.F.B. Haddad (pers comm)
Scinax hayii	Dynamic	C.F.B. Haddad (pers comm)
Scinax rizibilis	Dynamic	C.F.B. Haddad (pers comm)
Scinax ruber	Ontogenetic	A. Lima (pers comm)
Taychynemis seychellensis	Ontogenetic	Nussbaum & Wu 1995
Triprion petasatus	Ontogenetic	Duellman 2001
Werneria preussi	Ontogenetic	Duellman & Trueb 1986

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CHAPTER 2

OVERSEAS DISPERSAL OF HYPEROLIUS REED FROGS FROM CENTRAL AFRICA TO THE OCEANIC ISLANDS OF SÃO TOMÉ AND PRÍNCIPE[2]

Abstract

To infer the colonization history of reed frog species endemic to the oceanic islands of São Tomé and Príncipe, *Hyperolius molleri* and *H. thomensis*, we quantified phylogeographical structure in the closely related *H. cinnamomeoventris* species complex, which is broadly distributed across continental Central Africa. We combined gene and species tree analyses to investigate diversity and divergence among *H. cinnamomeoventris* populations, identify the most likely dispersal route to the islands, and infer the order in which the islands were colonized. One of the endemics (*H. molleri*) is distributed on both islands and we quantified genetic divergence between populations. We recovered three clades in *H. cinnamomeoventris* corresponding to West-, North/East-, and South-Central Africa. The island endemics form a monophyletic group most closely related to the West-Central African *H. cinnamomeoventris* clade. Populations of *H. molleri* on São Tomé and Príncipe are reciprocally monophyletic at mitochondrial loci but nuclear gene trees do not support this divergence. Genetic structure in the *H. cinnamomeoventris* species complex

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coincides with biogeographical barriers identified in previous studies of Central African rain forest taxa. Individual gene tree and species tree analyses support a single dispersal event from the Ogooué or Congo River basins (West-Central Africa) to the island of São Tomé with subsequent divergence within São Tomé and dispersal to Príncipe.

2.1 Introduction

The ecological theory of island biogeography describes species richness on islands as an equilibrium between colonization and extinction, yet many lineages undergo extensive diversification within islands and these *in situ* speciation events contribute significantly to total island biodiversity (Gillespie 2004, Whittaker *et al*. 2008). New species arise on islands via two key processes: colonization with subsequent divergence from the source population or diversification of existing island species (MacArthur & Wilson 1963, Losos & Schluter 2000, Emerson & Kolm 2005, Ricklefs & Bermingham 2007). Although hundreds of studies address colonization and subsequent diversification in island systems, many classic investigations of island biogeography have focused on relatively young and remote archipelagos (e.g. the Hawaiian and Galapagos Islands; Gillespie & Baldwin 2010), with focal species that are either good dispersers across saltwater barriers (e.g. birds; Diamond 1969) or that have radiated extensively within a particular archipelago (e.g. *Anolis* lizards; Losos & Schluter 2000). Here we examine these same mechanisms within an old archipelago that hosts numerous endemic species from groups that are typically considered poor dispersers across saltwater barriers.

The oceanic islands of the Gulf of Guinea (São Tomé, Príncipe, and Annobón) are located on the Cameroon Volcanic Line a few hundred kilometers from the western coast of Central Africa. The islands are comparable in age to the Macaronesian archipelagos (Azores, Cape Verde, Canary, and Madeira Islands), ranging from approximately 5 (Annobón) to 13 (São Tomé) to 30 (Príncipe) Myr, and in the course of this extended history they have accumulated hundreds of endemic species including shrews, burrowing reptiles, and amphibians (Jones 1994) which typically do not cross saltwater barriers and are absent from most oceanic islands (Darwin 1859, De Balsac & Hutterer 1982, Vitt & Caldwell 2014). Gulf of Guinea endemic plants and animals are taxonomically disparate and many species occur on only a single island (Figueiredo 1994, Jones 1994). This pattern suggests that much of the endemic diversity on Gulf of Guinea islands results from recurrent colonization from the mainland with subsequent divergence from source populations rather than *in situ* diversification of existing island species. This is in contrast to patterns of diversification in the Macaronesian archipelagos where intra-island speciation can overshadow dispersal as islands mature (Emerson & Oromí 2005, but see Illera *et al*. 2012). Like the Macaronesian archipelagos (Juan *et al*. 2000), the Gulf of Guinea islands share a number of sister-species across taxonomic groups (Jesus *et al*. 2009, Melo *et al*. 2011, Miller *et al*. 2012), indicating that dispersal *within* the island chain may have been an important mechanism generating diversity (Emerson 2002).

The prevailing hypothesis for the presence of amphibians, reptiles, and shrews on the Gulf of Guinea islands is assisted dispersal via vegetation rafts that are swept down

major river drainages into the gulf following large rain events (De Balsac & Hutterer 1982, Measey *et al*. 2007). Vegetation rafts have been observed leaving major rivers in Africa and South America (King 1962, Renner 2004) and inventories of such rafts in the Amazon have recovered a diverse assemblage of terrestrial vertebrates including frogs, snakes, caecilians, and lizards (Iherring 1911, Archaval *et al*. 1979, Schiesari *et al*. 2003*)*. Three major rivers that flow into the Gulf of Guinea could potentially serve as sources for these rafts: the Niger originating in West Africa, the Congo originating in East-Central Africa, and the Ogooué originating in West-Central Africa (Figure 2.1a). Although the mouth of the Ogooué River is most proximate to the islands (approximately 250 km), currents in the Gulf of Guinea direct freshwater plumes from the Niger and Congo Rivers towards the islands (Richardson & Walsh 1986); therefore, vegetation rafts originating in West or East African drainages could feasibly reach the islands.

Several phylogenetic studies of African herpetofauna address the colonization history of amphibians and reptiles of São Tomé and Príncipe based on geographical distributions of mainland species most closely related to island endemics. Phylogenetic studies for six of the seven endemic amphibians identify putative sister taxa with distributions in East Africa (Wilkinson *et al*. 2003, Drewes & Wilkinson 2004, Uyeda *et al*. 2007, Measey *et al*. 2007, Loader *et al*. 2007, Zimkus *et al*. 2010) and invoke long-distance dispersal via the Congo River as a possible dispersal route to the islands. Studies of the islands' reptile fauna identify putative sister taxa in West (Fritz *et al*. 2011), Central (Jesus *et al*. 2005, Carranza & Arnold 2006, Jesus *et al*.

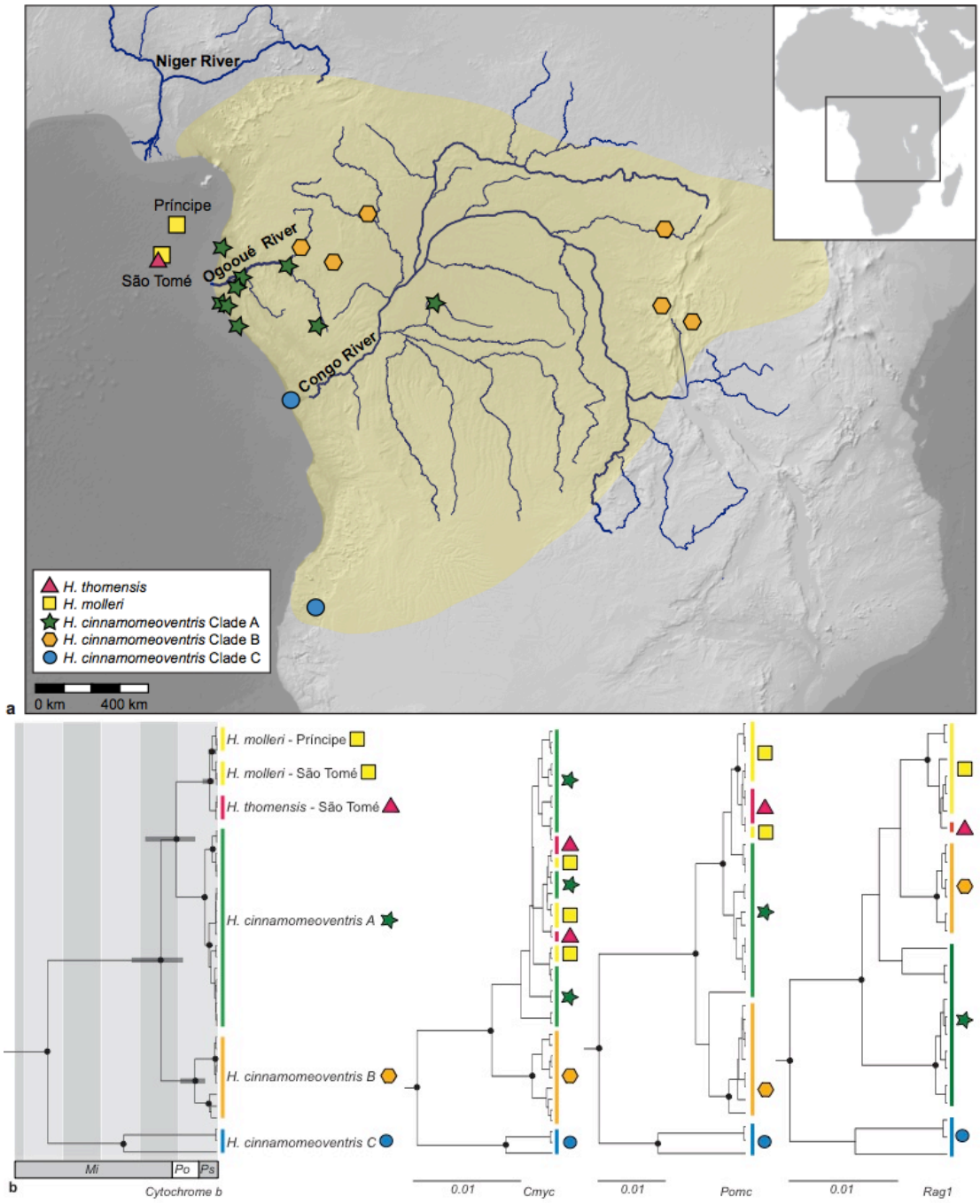

Figure 1 (a) Distribution of São Tomé and Príncipe island and Central African sampling localities. Sampling localities are colored according to mitochondrial clade and the clades supported by the *BEAST species tree analyses. The approximate range of the *H. cinnamomeoventris* species complex is shown in yellow. (b) Mitochondrial (*Cytochrome-b*) and nuclear (*Cmyc*, *Pomc*, *Rag1*) gene trees. 95% highest posterior density intervals for divergence time estimates discussed in the text are indicated on the *Cytochrome-b* gene tree. The axis indicates geological epochs Miocene (Mi), Pliocene (Po) and Pleistocene (Ps) and time before present in increments of five million years. Posterior probabilities greater than 0.95 are denoted by black dots.

2007), and East Africa (Jesus *et al*. 2005) indicating that dispersal from the Niger and Ogooué rivers may also be possible. These studies are largely limited by the availability of taxonomic sampling on the mainland, especially in the Congo River basin; therefore the frequency, timing, and origin of potential dispersal events, and consequently the evolutionary history of these enigmatic faunas, are poorly understood.

Among the islands' "poor dispersing" vertebrates, reed frogs (genus *Hyperolius*) are thought to be the only lineage that diversified within a single island and also dispersed between islands in the archipelago: *H. thomensis* Bocage is found only in forest habitats above 1000 m elevation on São Tomé and its putative sister taxon *H. molleri* (Bedriaga) is broadly distributed on both islands. Therefore, this sister-taxon pair presents an opportunity to jointly investigate colonization routes from the mainland, dispersal within the island chain, and *in situ* diversification. Their most closely related mainland congener is a widely distributed Central African species complex, the cinnamon-belly reedfrogs, *H. cinnamomeoventris* Bocage (Drewes & Wilkinson 2004, Schick *et al*. 2010), which inhabits moist savanna, bushland, forest clearings, and disturbed forest (Schiøtz 1999). The Central African distribution of the putative source species *a priori* rules out the Niger River as a colonization route; however, either the Congo or Ogooué rivers could serve as a potential *Hyperolius* dispersal route to the islands (Figure 2.1a). Here, we employ a multi-locus phylogeography approach with samples of *H. cinnamomeoventris* from throughout the species complex range to 1) investigate whether cryptic genetic diversity in *H. cinnamomeoventris* across Central

Africa coincides with biogeographical barriers identified in other Central African taxa, 2) determine whether *Hyperolius* on São Tomé and Príncipe result from a single colonization event from the mainland originating from either the Congo or Ogooué Rivers, and 3) quantify divergence between *H. molleri* populations on São Tomé and Príncipe.

2.2 Materials and Methods

2.2.1 Sampling details

Between 2001 and 2013 we collected 31 samples from 18 populations of the *Hyperolius cinnamomeoventris* species complex throughout Central Africa, three samples of *H. thomensis* from São Tomé, and six samples of *H. molleri* from São Tomé and Príncipe (Figure 2.1a). The *H. cinnamomeoventris* species complex extends to East Africa (Uganda and Western Kenya) but here we focused on the Central African portion of the range. Tissue samples (toe clips, liver or muscle) were preserved in 95% ethanol or RNAlater and specimens are accessioned in the Cornell University Museum of Vertebrates, California Academy of Sciences, North Carolina Museum of Natural Sciences, Smithsonian National Museum of Natural History, Museum für Naturkunde in Berlin, and National Museum in Prague, (Table 2.S1).

2.2.2 Laboratory methods

We extracted total genomic DNA using a DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA) and polymerase chain reaction (PCR)-amplified and sequenced one mitochondrial fragment (*cytochrome-b*) and three nuclear protein-coding genes

(*cmyc, pomc, Rag1*) using published primers (Table 2.1). PCRs were carried out in a final volume of 20μL containing: 20 ng template DNA, 1× Buffer, 0.2 μM of each primer, 0.4 mM dNTP mix, and 0.125 units of *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN, USA). Amplification was carried out with an initial denaturation for 5 min at 94 °C, followed by 35 cycles (60 s denaturation at 94 °C, 60 s annealing at 42-55°C (Table 2.1), 60 s extension at 72 °C), and a final extension at 72 °C for 5 min. PCR products were purified using ExoSAP-IT (USB Corp., Cleveland, OH, USA) and sequenced using a BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI Automated 3730xl Genetic Analyzer (Applied Biosystems). DNA sequences were edited using SEQUENCHER 5.0.1 (Gene Codes Corp., Ann Arbor, MI, USA) and are accessioned in GenBank (KJ865916-KJ866052).

2.2.3 Mitochondrial and nuclear gene tree estimation

To investigate patterns of mitochondrial and nuclear divergence across the range of *H. cinnamomeoventris* and among the island endemics, we generated gene trees for *cytochrome b* and the three nuclear loci. Sequences were aligned using CLUSTAL X 2.0.10 (Larkin *et al*. 2007). We verified the absence of recombination within nuclear loci using the sum of squares method in TOPALi 2 (Milne *et al*. 2008) and used PARTITIONFINDER 1.1.0 (Lanfear *et al*. 2012) to establish that the HKY+G model and TrN+G model (not partitioned by codon position) best represented substitution processes for the mitochondrial and each of the nuclear fragments, respectively. We estimated gene trees for each locus using Bayesian phylogenetic analyses

Table 2.1 Primer sequences and amplification conditions for mitochondrial and nuclear sequences collected from *Hyperolius molleri* (São Tomé and Príncipe Islands), *H. thomensis* (São Tomé Island), and the *H. cinnamomeoventris* species complex (Central Africa). * indicates 0.3 μL of additional MgCl per reaction

Primer Sequence	Locus (bp)	PCR Annealing Temperature *HC*	*HM*	*HT*	Reference
MVZ15 5' GAA CTA ATG GCC CAC ACW WTA CG 3'	616	43*	42*	42*	(Moritz *et al*, 1992)
MVZ16 5' AAA TAG GAA RTA TCA YTC TGG TTT RAT 3'					(Moritz *et al*. 1992)
CMYC 1U 5' GAG GAC ATC TGG AAR AAR TT 3'	434	48	49	48	(Crawford 2003)
CMYC ex2dR 5' TCA TTC AAT GGG TAA GGG AAG ACC 3'					(Wiens *et al*. 2005)
POMC1 5' GAA TGT ATY AAA GMM TGC AAG ATG GWC CT 3'	521	55*	52*	52*	(Wiens *et al*. 2005)
POMC2 5' TAY TGR CCC TTY TTG TGG GCR TT 3'					(Wiens *et al*. 2005)
Rag1 F 5' GCC AGA TCT TTC ARC CAC TC 3'	467	55*	50*	52	*L.P. Lawson*
Rag1 R 5' TGA TCT CTG GAA CRT GGG CTA 3'					*(Pers comm.)*

implemented in BEAST 1.8.0 (Drummond *et al*. 2012) with a constant size coalescent tree prior and a strict molecular clock model. For each tree we obtained posterior distributions from two independent Markov chain Monte Carlo (MCMC) simulations, each run for 10 million generations, and assessed convergence with TRACER 1.5 (Rambaut *et al*. 2013). The effective sample size for each parameter was well above 200 and simulations were repeated without sequence data to test the influence of priors on posterior distributions for all parameters. We combined tree files from replicate runs using LOGCOMBINER and discarded the first 10% of trees as burn-in prior to summarizing the posterior distribution of trees using TREEANNOTATOR. No fossils of hyperoliid frogs exist with which to calibrate divergence times for our gene trees, therefore we applied a constant rate of sequence divergence estimated for mitochondrial genes in tropical bufonid frogs (0.80 - 1.90% per Myr; Sanguila *et al*. 2011) to *cytochrome b*. We selected a rate prior with a mean of 1.4% and a normal distribution (95% confidence interval of 0.8 – 1.9%).

We used ARLEQUIN 3.1 (Excoffier *et al*. 2005) to calculate nucleotide diversity based on number of segregating sites (θs) and on pairwise sequence comparisons ($\theta \pi$) for clades in our topology, as well as uncorrected and net sequence divergence (*Dxy* and *Da* using the Tamura–Nei model; Tamura & Nei 1993), and F_{ST} between clades.

2.2.4 Species tree reconstruction

Individual gene trees may differ from the underlying species tree (Maddison 1997), so we used the multi-coalescent model implemented in *BEAST (Heled & Drummond

2010) to infer a species tree for the island and mainland *Hyperolius* clades using the three nuclear loci. This method assumes lineage sorting is the main source of inconsistency between gene trees and the underlying species tree, no recombination within loci, no gene flow between species post-divergence, and requires the prior assignment of individuals to putative species. The current species designations in our study taxa are based on morphological characters and do not necessarily reflect true diversity or evolutionary relationships; therefore, we assigned individuals to putative species for the *BEAST analysis following the geographical clades recovered in the mitochondrial gene tree (Figure 2.1b, Table 2.S1). We resolved haplotypes for heterozygous individuals using PHASE v 2.1 (Stephens *et al*. 2001) implemented in DnaSP v 5.1 (Librado & Rozas 2009). The *BEAST analysis only included samples with sequence data for at least two of the three nuclear loci and we specified unlinked site, clock, and tree models, a Yule process tree prior, and a strict molecular clock model with *cmyc* as the reference gene (clock rate set to 1). We obtained posterior distributions from two independent MCMC simulations, each run for 100 million generations and assessed convergence and the influence of priors as described above for gene trees. The species tree was inferred as a maximum clade credibility tree with node ages represented by median heights.

2.3 Results

2.3.1 Mitochondrial and nuclear gene trees

The mitochondrial gene tree reveals three distinct clades of *Hyperolius cinnamomeoventris* corresponding to a West- (Clade A), a North/East- (Clade B), and

a South-Central African clade (Clade C; Figure 2.1b). The two island species, *H. thomensis* and *H. molleri*, form three distinct clades (São Tomé and Príncipe *H. molleri* populations are reciprocally monophyletic; *Da* = 1.5%) that are nested within *H. cinnamomeoventris* and share a most recent common ancestor with the West-Central African clade. The island clades are significantly differentiated from mainland clades (*Da* = 8.8-15.5%, F_{ST} = 0.615-0.737; Table 2.2) and genetic diversity is greater in mainland than in island clades (Table 2.3).

The three nuclear loci reveal partly conflicting relationships among the mainland and island mitochondrial clades. Both *cmyc* and *pomc* support the West-Central African clade (Clade A) as sharing a most recent common ancestor with the island species. In contrast, the *Rag1* gene tree shows insufficient support to differentiate between the West-Central (Clade A) and North/East-Central African (Clade B) clades as sharing a most recent common ancestor with the island species (Figure 2.1b). Additionally, the nuclear gene trees indicate substantial incomplete lineage sorting among Clade A, Clade B and the island endemics (Figure 2.1b). The island species are undifferentiated at nuclear loci but exhibit moderate divergence from the West-Central African *H. cinnamomeoventris* (Clade A; *Da* = 0.2-0.4%, F_{ST} = 0.411-0.640; Table 2.2) and significant divergence from the two remaining clades (*Da* = 1.0-2.8%, F_{ST} = 0.789-0.900; Table 2.2).

2.3.2 Species tree reconstruction

Table 2.2 Estimates of pairwise F_{ST} values between *Hyperolius molleri* (São Tomé and Príncipe Islands), *H. thomensis* (São Tomé Island), and the three Central African clades of the *H. cinnamomeoventris* species complex (Clades A, B and C) for *cytochrome-b* (mtDNA) and combined nuclear loci (nuDNA). Values significant at the 0.05 level are shown in bold.

	H. molleri Príncipe	*H. molleri* São Tomé	*H. thomensis* São Tomé	*H. cinnamomeoventris* A	*H. cinnamomeoventris* B
H. moll São Tomé (mtDNA)	0.934				
H. moll São Tomé (nuDNA)	0.750	--			
H. thom São Tomé (mtDNA)	0.979	0.980			
H. thom São Tomé (nuDNA)	0.750	0.000	--		
H. cinn A (mtDNA)	**0.726**	**0.717**	**0.728**		
H. cinn A (nuDNA)	**0.640**	**0.595**	**0.411**	--	
H. cinn B (mtDNA)	**0.619**	**0.615**	**0.632**	**0.609**	
H. cinn B (nuDNA)	**0.900**	**0.900**	**0.880**	**0.797**	--
H. cinn C (mtDNA)	**0.737**	0.727	0.729	**0.778**	**0.645**
H. cinn C (nuDNA)	0.811	0.814	0.789	**0.855**	**0.885**

Table 2.3 Summary statistics for mitochondrial and nuclear loci collected from *Hyperolius molleri* (São Tomé and Príncipe Islands), *H. thomensis* (São Tomé Island), and the three clades of the *H. cinnamomeoventris* species complex (Central Africa).

	mtDNA					nuDNA			
	N	bp	N_h	θs	θπ	N	bp	θs	θπ
H. cinnamomeoventris Clade A	18	616	12	0.0486	0.0373	19	1419	0.0050	0.0022
H. cinnamomeoventris Clade B	8	616	6	0.0695	0.0675	9	1419	0.0023	0.0016
H. cinnamomeoventris Clade C	3	616	2	0.1158	0.1158	3	1419	0.0132	0.0127
H. molleri Príncipe	3	616	2	0.0011	0.0011	3	1419	0.0005	0.0005
H. molleri São Tomé	3	616	2	0.0011	0.0011	3	1419	0.0000	0.0000
H. thomensis São Tomé	3	616	1	0.0000	0.0000	3	1419	0.0000	0.0000

N, number of samples, bp, sequence length in base pairs; N_h, number of haplotypes; θs, genetic diversity based on the number of segregating sites; θπ, genetic diversity based on pairwise sequence comparisons.

We assigned samples to operational species units following the geographical clades recovered in the mitochondrial gene tree (Figure 2.1b). There were only a few variable sites present between the recently diverged island clades therefore we grouped samples of the island endemic species (*H. thomensis* and *H. molleri*) into a single clade in the species tree analysis. The species tree reconstruction strongly supports *H. cinnamomeoventris* Clade A and the island *Hyperolius* as sister taxa (Figure 2.2).

2.4 Discussion

2.4.1 Cryptic genetic diversity in H. cinnamomeoventris

The *Hyperolius cinnamomeoventris* species complex is distributed in disturbed forest, moist savanna, and bushland habitats across Central Africa and is hypothesized to consist of several cryptic species (Lötters *et al*. 2004, Schick *et al*. 2010). We recover substantial genetic diversity across the species range, consistent with a previous mitochondrial study that identified four regional clades in the *H. cinnamomeoventris* species complex, including *H. veithi,* a newly described species from the central Congo Basin (Schick *et al*. 2010). We identify three distinct clades (not including *H. veithi*) across the Central African range of *H. cinnamomeoventris* that correspond to West-Central, North/East-Central, and South-Central Africa (Clades A through C, respectively). The biogeographical break between West-Central and North-Central Africa is old (Middle to Late Miocene, approximately 12.3 to 4.9 Ma; Figure 2.1b) and is consistent with studies in several rain forest plants that identify distinct lineages along the border of Cameroon and Gabon (reviewed in Hardy *et al*. 2013). This region roughly coincides with the climatic hinge, a zone of North-South seasonal inversion at

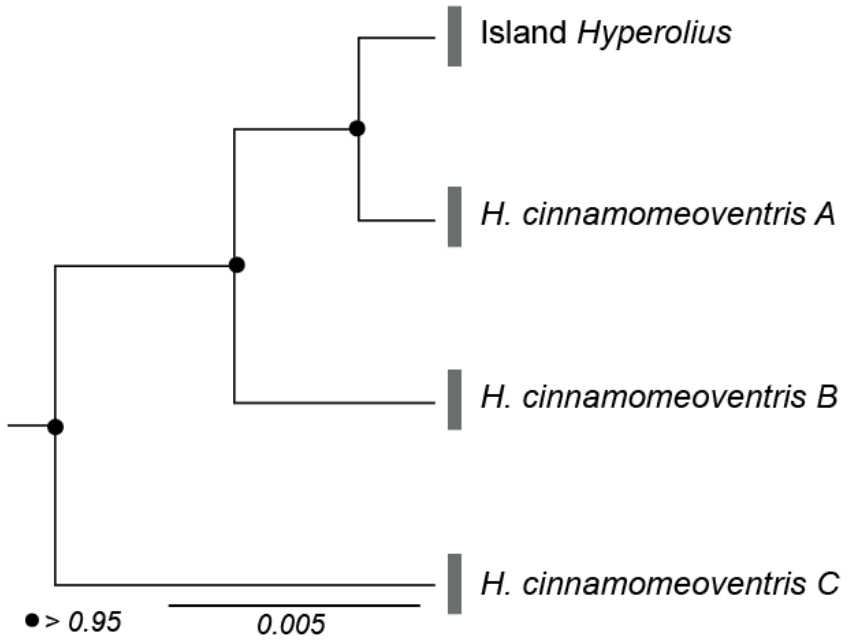

Figure 2.2 * BEAST species tree inference for nuclear (*Cmyc*, *Pomc*, *Rag1*) haplotypes collected from *Hyperolius thomensis*, *H. molleri* and the *H. cinnamomeoventris* species complex from Central Africa and the islands of São Tomé and Príncipe. Posterior probabilities greater than 0.95 are denoted by black dots.

which climates transition from boreal to austral and where the severity and duration of the dry season increase with latitude (Suchel 1990). One potential mechanism for divergence along this gradient is long-term reduction in gene flow across the climatic hinge due to differences in breeding phenology (Hardy *et al*. 2013).

Alternatively, the biogeographical break may result from expansion following periods of isolation in climatic refugia north and south of the climatic hinge (Hardy *et al*. 2013). Lineage diversification among invertebrate and small vertebrate taxa due to population expansion and contraction through Pliocene-Pleistocene climatic cycles is well documented in tropical rain forests outside Africa such as the Atlantic Coastal Forest of Brazil (Carnaval *et al*. 2009) and the Australia Wet Tropics rain forest (Moritz *et al*. 2009). Similarly, the Guineo-Congolian rain forests expanded and contracted throughout the Pliocene and Quaternary in response to global glacial cycles (Plana 2004) and thus climatic refugia may play an important role in generating taxonomic diversity and shaping current genetic structure within species (Quérouil *et al*. 2003, Tosi 2008, Nicolas *et al*. 2010, Leaché & Fujita 2010, Born *et al*. 2010, Johnston & Anthony 2012). Patterns of endemism and fine-scale phylogeographical structure in several rain forest taxa are consistent with the persistence of lineages in a central refuge in the west-central Congo Basin and multiple smaller refugia throughout western Central Africa (Tosi 2008, Nicolas *et al*. 2010, Born *et al*. 2010, Hardy *et al*. 2013). As in other forest taxa, the presence of fine scale genetic structure within Clade A of *H. cinnamomeoventris* in Gabon is consistent with lineage persistence in several small refugia during more recent periods of aridification in this region (Nicolas *et al*.

2010, Born *et al*. 2010). Likewise, relatively deep divergence between eastern and western populations within Clade B (estimated at the Pliocene-Pleistocene transition; Figure 2.1b) may reflect persistence in climatic refugia on either side of the Congo basin with recent recolonization following rain forest expansion. Although our current sampling does not permit us to directly test this hypothesis, several studies cite genetic exchange across the northern Congo basin during more humid periods as a key mechanism shaping the distribution and diversity of plants, birds, and primates in Central Africa (Fjeldså & Lovett 1997, Tosi 2008). Finally, the samples comprising Clade C are highly divergent from remaining clades and indicate previously unrecognized diversity in the South-Central African range of *H. cinnamomeoventris*, which may contain several independent lineages.

2.4.2 Dispersal to São Tomé and Príncipe

The gene trees reveal that the island endemic *Hyperolius* form a clade that renders *H. cinnamomeoventris* paraphyletic, consistent with a previous mitochondrial phylogenetic study (Schick *et al*. 2010). This result confirms that a clade within the *H. cinnamomeoventris* species complex is the sister taxon to the island endemics and indicates that island endemics likely resulted from one colonization from the mainland and subsequent diversification within the Gulf of Guinea. Because we identified three geographical clades in *H. cinnamomeoventris* that largely correspond to the Ogooué and Western Congo River Basins (Clade A), the North-Eastern Congo River Basin (Clade B), and South-Central Africa (Clade C), we can differentiate among potential dispersal routes to the islands. The mitochondrial topology and two of the three

nuclear gene trees support a sister relationship between the West-Central African clade of *H. cinnamomeoventris* (Clade A) and the island endemics, while the remaining nuclear gene tree supports either the West-Central or North-East clade, as sister to the island endemics. The *BEAST species tree analysis strongly supports a West-Central African origin for the source population, thus the predominant pattern indicates that *Hyperolius* likely dispersed to the islands on a vegetation raft that originated along the Ogooué River or the western extent of the Congo River. We estimate divergence between the island endemics and the West-Central African clade of *H. cinnamomeoventris* in the Late Miocene to Early Pliocene (approximately 8.9 to 3.4 Ma), a period during which glacial cycles shifted species distributions in the Guineo-Congolian region (Plana 2004). Additional phylogeographic sampling coupled with bioclimatic modelling of the *H. cinnamomeoventris* distribution under past climatic regimes may refine our understanding of the West-Central African clade's distribution in the Late Miocene/Early Pliocene and differentiate between the Ogooué or Western Congo Rivers as a more likely dispersal route to the islands.

Despite evidence that a freshwater plume extends from the mouth of the Ogooué River to São Tomé (Jourdin *et al*. 2006), the Ogooué has not previously been identified as a potential source for vegetation rafts reaching the Gulf of Guinea islands. A phylogenetic study of Newton's grassland frog, *Ptychadena newtoni* (Bocage), endemic to the island of São Tomé found strong support for a sister relationship with the *P. mascareniensis* species complex (Duméril and Bibron), which is broadly distributed across sub-Saharan Africa, North-East Africa, Madagascar and the

Seychelles (Measey *et al*. 2007). The island endemic clusters with samples from East and North-East Africa in the mitochondrial phylogeny, so the authors invoked an East African origin of dispersal and highlight the Congo River as a likely dispersal route to the islands, however, sampling of *P. mascareniensis* is rather limited and none were available from West-Central Africa or the Congo Basin. Likewise, a phylogenetic study of island *Phrynobatrachus* Günther puddle frogs [*P. dispar* (Peters), endemic to Príncipe and *P. leveleve* Uyeda, Drewes, and Zimkus, endemic to São Tomé] placed the island endemics in a South and East African clade (Zimkus *et al*. 2010) but interpretation of this pattern is limited by low phylogenetic resolution and geographically limited sampling. Although considerable evidence supports that dispersal from East Africa to the Gulf of Guinea along the Congo River is possible (Jourdin *et al*. 2006, Measey *et al*. 2007), more detailed studies of cryptic diversity in widespread mainland species are needed to identify the timing and origin of such events and further refine the role of vegetation rafts in the colonization history of São Tomé and Príncipe's endemic amphibians.

2.4.3 Diversification within the Gulf of Guinea Islands

Our phylogeographical analyses are consistent with a single dispersal event to the Gulf of Guinea with subsequent diversification within the island chain. The mitochondrial gene tree strongly supports *H. molleri* and *H. thomensis* as distinct clades but this divergence is not supported by nuclear gene topologies, which is expected given the larger effective population size of nuclear loci (Birky *et al*. 1989, Ballard & Whitlock 2004) and the relatively recent divergence between these species (estimated between

1.7 and 0.5 Ma; Figure 2.1b). The current distributions of the two species on São Tomé are partly sympatric at mid-elevations and the species differ in breeding biology (*H. thomensis* breeds exclusively in water-filled tree hole cavities whereas *H. molleri* breeds near still or slow-moving water) and in morphology (*H. thomensis* is nearly twice the size of *H. molleri*); multiple lines of evidence thus support recognizing these endemics as distinct species. Divergence between the São Tomé and Príncipe populations of *H. molleri* is estimated to be more recent at approximately 1.1 Myr to 270 kyr. Although the two populations are reciprocally monophyletic in the mitochondrial gene tree, this divergence is not supported by nuclear gene topologies. The pattern of mitochondrial divergence between these three clades, however, implies that *Hyperolius* colonized São Tomé first, that the founding population diverged *in situ* to form *H. thomensis* and *H. molleri*, and that *H. molleri* subsequently colonized Príncipe.

Our results corroborate that the distribution of *H. molleri* on São Tomé and Príncipe results from dispersal between the two islands as opposed to independent colonization events from the mainland. Dispersal between São Tomé, Príncipe, and Annobón is fairly common in angiosperms (Figueiredo 1994) and more mobile animals such as birds, snakes and lizards (Jesus *et al*. 2009, Melo *et al*. 2011, Miller *et al*. 2012) but *H. molleri* is the only amphibian known to have successfully dispersed between the islands. Although two species of *Phrynobatrachus* are endemic to the Gulf of Guinea, divergence between *P. leveleve* (São Tomé) and *P. dispar* (Príncipe) likely predates the estimated age of São Tomé (13 Myr; Lee *et al*. 1994) and the species are not each

others' closest relatives (Zimkus *et al*. 2010). Therefore, the endemic *Phrynobatrachus* likely resulted from independent colonization events from continental Africa (Uyeda *et al*. 2007).

Vegetation rafts may facilitate amphibian dispersal between the two islands, but we expect that such events are relatively uncommon as our mitochondrial phylogeny indicates that gene flow between the two islands is likely not ongoing. Future studies of historical population demography of *H. molleri* may provide additional insight into the colonization history of the islands including more precise estimates of timing of dispersal and the approximate size of the founding population. Furthermore, because dispersal between the two islands occurred relatively recently, *H. molleri* present an opportunity to investigate the effects of small founding population size and ecological selection on the early stages of genotypic and phenotypic divergence.

Mounting evidence supports the significant role of long-distance dispersal in shaping global patterns of biogeography and the accumulation of biodiversity on oceanic islands (de Queiroz 2005, Cowie & Holland 2006), even in taxonomic groups that are considered poor dispersers across saltwater barriers (Vences *et al*. 2003, Vidal *et al*. 2008, Maddock *et al*. 2014). The Gulf of Guinea islands present an ideal system in which to quantify the relative contributions of dispersal versus *in situ* diversification in generating biodiversity in an older archipelago (Juan *et al*. 2000, Emerson & Oromí 2005, Kim *et al*. 2008, Illera *et al*. 2012).

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SUPPLEMENTAL INFORMATION FOR CHAPTER 2 - OVERSEAS DISPERSAL OF HYPEROLIUS REED FROGS FROM CENTRAL AFRICA TO THE OCEANIC ISLANDS OF SÃO TOMÉ AND PRÍNCIPE

Table 2.S1 Sampling localities and voucher information. Country abbreviations as collow: Angola (AO) Cameroon (CM) Democratic Republic of the Congo (CD) Gabon (GA) Republic of Congo (RC) Rwanda (RW) São Tomé and Príncipe (ST&P). Museum abbreviations as follow: Cornell University Museum of Vertebrates (CUMV), the California Academy of Sciences (CAS), the North Carolina Museum of Natural Sciences (NCSM), the Smithsonian National Museum of Natural History (USNM), Museum für Naturkunde, Berlin (ZMB), and the National Museum in Prague (NMP). Samples without catalogue numbers are in personal collections: A. Channing (AC), E. Greenbaum (EBG), V. Gvoždík (VG), Z.T. Nagy (PM).

Species	Country	Locality	Catalogue No.	Field No	Lat	Long
H. cinn	AO	Huila Province, Humpata	NA	AC3096	-14.98	13.43
H. cinn	AO	Huila Province, Humpata	NA	AC3097	-14.98	13.43
H. cinn	CM	East Province, Malapa	NMP6V 74716	VG10194	2.10	15.36
H. cinn	CD	Bandundu, Gongo-Yembe	NA	VGCD1273	-1.92	18.64
H. cinn	CD	Bandundu, Gongo-Yembe	NA	VGCD1274	-1.92	18.64
H. cinn	CD	Bas-Congo, Muanda	NA	PM035	-5.92	12.35
H. cinn	CD	Bas-Congo, Luango-Nzambi	NA	PM056	-5.89	12.77
H. cinn	CD	Bas-Congo, Luango-Nzambi	NA	PM058	-5.89	12.77
H. cinn	CD	North Kivu	NA	EBG2305	1.40	28.57
H. cinn	CD	South Kivu	NA	EBG1306	-1.87	28.45
H. cinn	GA	Estuaire, Sahoué	NMNH 578128	NMNH578128	0.60	9.34
H. cinn	GA	Estuaire, Sahoué	NMNH 578129	NMNH578129	0.60	9.34
H. cinn	GA	Moyen-Ogooué, Lac Oguemoué	NCSM 81282	BLS16228	-1.12	10.03
H. cinn	GA	Moyen-Ogooué, Lac Oguemoué	CAS 254490	BLS16229	-1.12	10.03
H. cinn	GA	Moyen-Ogooué, Lambaréné	NCSM 81280	BLS16215	-0.69	10.23
H. cinn	GA	Moyen-Ogooué, Lambaréné	NCSM 81281	BLS16216	-0.69	10.23
H. cinn	GA	Nyanga, Gamba	NMNH 578115	NMNH578115	-2.79	10.05
H. cinn	GA	Nyanga, Gamba	NMNH 578116	NMNH578116	-2.79	10.05
H. cinn	GA	Ogooué-Ivindo, Ipassa Station	CUMV14954	BLS13800	0.51	12.80
H. cinn	GA	Ogooué-Ivindo, Ipassa Station	CUMV14955	BLS13801	0.51	12.80
H. cinn	GA	Ogooué-Ivindo, Ipassa Station	CUMV15028	BLS14018	0.51	12.80
H. cinn	GA	Ogooué-Ivindo, Ivindo	CUMV15495	BLS14714	-0.21	12.29
H. cinn	GA	Ogooué-Ivindo, Ivindo	CUMV15498	BLS14717	-0.21	12.29
H. cinn	GA	Ogooué-Ivindo, Ivindo	CUMV15518	BLS14744	-0.20	12.20
H. cinn	GA	Ogooué-Maritime, Iguela	CUMV15092	BLS14236	-1.81	9.36
H. cinn	GA	Ogooué-Maritime, Rembo Rabi	CUMV15105	BLS14257	-1.89	9.57
H. cinn	RC	Cuvette-Ouest, Otsouandjoko	NA	VGCG12093	0.07	14.24
H. cinn	RC	Cuvette-Ouest, Otsouandjoko	NA	VGCG12092	0.07	14.24
H. cinn	RC	Lekoumou, Kissiki	USNM 584159	FSKJ246971	-2.79	13.54
H. cinn	RC	Lekoumou, Kissiki	USNM 584160	FSKJ246979	-2.79	13.54
H. cinn	RW	Southern Province, Butare	ZMB 77533	JMD651	-2.60	29.74
H. moll	ST&P	Príncipe, Baie das Agulahs	CAS 219203	CAS219203	1.60	7.35
H. moll	ST&P	Príncipe, Chada Agua Doutor	CAS 219128	CAS219128	1.65	7.42
H. moll	ST&P	Príncipe, Papagio River	CAS 233492	CAS233492	1.63	7.42
H. moll	ST&P	São Tomé, Lagoa Amélia	CAS 219055	CAS219055	0.29	6.60
H. moll	ST&P	São Tomé, Caxueira	CAS 218850	CAS218850	0.30	6.73
H. moll	ST&P	São Tomé, Java	CAS 218974	CAS218974	0.26	6.65
H. thom	ST&P	São Tomé, Bom Socesso	CAS 218929	CAS 218929	0.28	6.61
H. thom	ST&P	São Tomé, Bom Socesso	CAS 218934	CAS 218934	0.28	6.61
H. thom	ST&P	São Tomé, Bom Socesso	CAS 233475	CAS 233475	0.28	6.61

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CHAPTER 3

EVOLUTIONARY GENOMICS OF DIVERSIFICATION IN THE GULF OF GUINEA: DISPERSAL AND IN SITU SPECIATION IN AFRICAN REED FROGS

Abstract

Both organismal traits and island characteristics mediate the relative importance of dispersal versus *in situ* speciation in generating diversity on oceanic islands. *In situ* speciation is typically restricted to larger and environmentally diverse islands that allow for allopatric divergence, however divergent ecological selection on islands may drive speciation in spite of gene flow. We use population genomic approaches to characterize inter-island dispersal and *in situ* speciation in reed frogs endemic to the Gulf of Guinea islands. Using mitochondrial sequence and genome-wide SNP data we demonstrate that amphibian dispersal in the Gulf of Guinea proceeded from the younger island (São Tomé) to the older island (Príncipe) indicating that for organisms that disperse overseas on rafts, dispersal between islands may largely be determined by ocean currents and not island age. We find that dispersal between the islands is not ongoing and that divergence in allopatry has resulted in genotypically distinct but phenotypically similar lineages on the two islands. Likewise, *in situ* diversification on São Tomé likely proceeded in allopatry due to the geographic separation of available breeding sites, but divergent ecological selection has resulted in genotypically and phenotypically distinct species. We find evidence of extensive hybridization between the two species where their ranges are currently sympatric, and the hybrid zone

coincides with a transition from agricultural land to primary forest suggesting that recent anthropogenic development may have enabled secondary contact between previously allopatric species.

3.1 Introduction

Oceanic islands accumulate endemic species via two key mechanisms: colonization by continental or adjacent island species that subsequently diverge from source populations or *in situ* diversification of resident island species (MacArthur & Wilson 1963, Heaney 2000, Whittaker *et al*. 2008). The relative importance of dispersal versus *in situ* speciation in generating diversity on islands varies predictably with a number of characteristics particular to each archipelago; the contribution of *in situ* diversification increases with island size (Losos & Schluter 2000, Parent *et al*. 2008), age (Emerson & Oromí 2005), and remoteness (Gillespie & Roderick 2002) whereas dispersal dominates in archipelagos with numerous small and young islands that are near continental sources (Paulay 1994). Organismal traits also mediate the contributions of dispersal and *in situ* speciation to overall rates of diversification within an archipelago; organisms with typically low dispersal abilities but a tendency for passive long-distance dispersal display high rates of inter-island colonization and diversification (e.g. land snails; Chiba 1999, Parent *et al*. 2008) whereas those with limited vagility or rapid divergence in secondary sexual traits provide more opportunities for divergent ecological or sexual selection to drive speciation within an island (Paulay 1985, Mendelson & Shaw 2005).

Most *in situ* diversification on islands proceeds via allopatric speciation and is therefore typically limited to larger islands (Coyne & Price 2000, Losos & Schluter 2000, Parent & Crespi 2006, Kisel & Barraclough 2010) because they offer more opportunities for geographic isolation (Endler 1977, Rosenzweig 1995) and tend to have greater altitudinal variation and habitat diversity (Ricklefs & Lovette 1999, Ackerman *et al*. 2007, Losos & Parent 2009). In some cases, *in situ* diversification may proceed via sympatric speciation (i.e. with gene flow), particularly on smaller islands where there are fewer opportunities for geographic isolation (Savolainen *et al*. 2006). While the sympatric distribution of closely related yet phenotypically disparate species is suggestive of sympatric speciation via divergent ecological selection (e.g. character displacement; Brown & Wilson 1956, Slatkin 1980), phenotypic differences that arise in allopatry may permit closely related species to coexist in secondary sympatry (Gillespie *et al*. 1997, Losos 2009); thus differentiating between the two processes can be challenging, even in model cases (Stuessy 2006). Here we use population genomic approaches to characterize the relative roles of inter-island dispersal and mechanisms of *in situ* speciation shaping diversification in reed frogs endemic to the Gulf of Guinea islands.

The Gulf of Guinea archipelago is located on the Cameroon Volcanic Line a few hundred kilometers from the western coast of Central Africa and comprises one land-bridge island (Bioko) and three oceanic islands (São Tomé, Príncipe, and Annobón). The oceanic islands have remained isolated from continental Africa throughout their history, yet because they are relatively old, ranging from approximately 5 (Annobón)

to 13 (São Tomé) to 30 (Príncipe) Myr, they have accumulated hundreds of endemic species (Jones 1994). Due to the high taxonomic diversity of island endemics, and close proximity of the islands to coastal Africa, dispersal from the mainland to the islands has been proposed as a key mechanism shaping patterns of diversity in the archipelago (Jones 1994, Measey *et al*. 2007). Furthermore, the islands share a number of sister-species across taxonomic groups, indicating that inter-island dispersal *within* the island chain may have been an important mechanism generating diversity in the archipelago (Jesus *et al*. 2009, Melo *et al*. 2011, Miller *et al*. 2012, Bell *et al*. *in review*). Although the islands are small, ranging in size from approximately 18 (Annobón) to 136 (Príncipe) to 850 (São Tomé) km^2, some lineages may have diversified rapidly within a single island to fill divergent ecological niches (Melo *et al*. 2011). However, mechanisms driving *in situ* diversification, as well as the relative contributions of dispersal versus *in situ* diversification in shaping total diversity, remain poorly characterized.

Among the islands' endemic vertebrates, reed frogs (genus *Hyperolius*) are thought to be one of the only lineages that diversified within a single island and also dispersed between islands in the archipelago (Jones 1994, Bell *et al*. *in review*). Therefore this lineage provides an opportunity to jointly investigate mechanisms driving *in situ* diversification as well as the frequency and demographic consequences of inter-island dispersal. The São Tomé Giant Reed frog (*H*. *thomensis*) is found only in forest habitats above 1000 m elevation on São Tomé and its sister taxon *H*. *molleri* is broadly distributed on both islands, occurring up to 1400 m elevation on São Tomé

and up to the summit on Príncipe (900 m). *Hyperolius thomensis* and *H. molleri* are considered distinct species based on differences in body size, coloration, and breeding ecology (Drewes & Wilkinson 2004), but individuals exhibiting intermediate phenotypes are found between 1000-1400 m where the species' ranges overlap on São Tomé (Bell & Drewes, unpublished data). This observation implies some level of gene flow between the two species; either throughout their evolutionary history (i.e. divergence in sympatry), or more recently as a consequence of range expansions in one or both species (i.e. divergence in allopatry with secondary contact). In contrast, although the presence of *H. molleri* on both São Tomé and Príncipe indicates that successful dispersal between the islands occurred at least once, these populations are reciprocally monophyletic at mitochondrial loci (mtDNA; Bell *et al. in review*). Therefore, we expect that dispersal events between the islands are relatively uncommon and that populations of *H. molleri* on Príncipe diverged allopatrically from those on São Tomé.

A recent multi-locus phylogeography study of the island *Hyperolius* and their mainland sister taxon (*H. cinnamomeoventris*) indicated that *Hyperolius* dispersed from West-Central Africa approximately 8.9 to 3.4 Mya and subsequently diversified within the archipelago (Bell *et al. in review*). In most archipelagoes, dispersal and colonization proceed from older to younger islands, following the "progression rule" (Wagner & Funk 1995, Roderick & Gillespie 1998, Juan *et al.* 2000), but patterns of mtDNA divergence among the island lineages suggested initial colonization of São Tomé (the younger of the two islands), *in situ* diversification on São Tomé resulting in

H. thomensis and *H. molleri*, followed by dispersal of *H. molleri* to Príncipe. Further inferences on the frequency of inter-island dispersal and the context of *in situ* diversification were limited due to small sample sizes and because the island lineages were undifferentiated at the slowly evolving nuclear coding genes (nuDNA) used in that study (Bell *et al. in review*). Here we combine mitochondrial phylogeography and genome-wide SNP data with population level sampling of the island species to 1) determine whether dispersal and colonization within the archipelago is an exception to the progression rule, 2) quantify the extent of inter-island dispersal and ensuing diversification, and 3) characterize the temporal and geographic extent of gene flow between sister species in a case of *in situ* diversification.

3.2 Material and Methods

3.2.1 Sampling details

Although *H. molleri* is distributed on both islands, it is currently considered a single species and we refer to the genetically distinct populations as the São Tomé and Príncipe lineages of *H. molleri* for clarity. Between 2001 and 2013 we collected 97 samples from 20 populations of *Hyperolius molleri* throughout its range on the islands of São Tomé and Príncipe, 20 samples from two populations of *H. thomensis* on São Tomé, and six samples from four populations of *H. cinnamomeoventris* from Gabon in continental Central Africa (Figure 3.1). For sites between 1000 and 1400 m elevation on São Tomé where *H. molleri* and *H. thomensis* are sympatric and potentially hybridizing, we preliminarily classified individuals according to differences in body size between the two species [*H. thomensis* male snout-vent-length (SVL) > 33 mm,

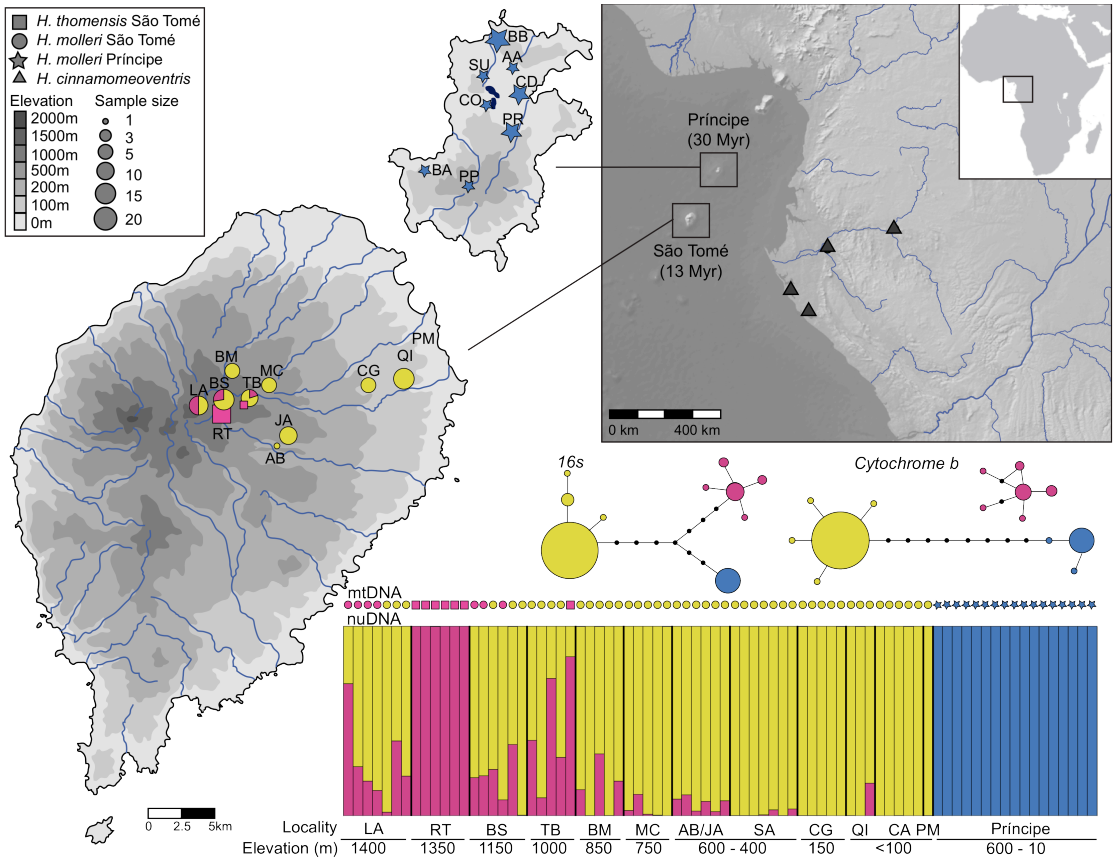

Figure 3. 1 Sampling localities on the islands of São Tomé and Príncipe (*Hyperolius thomensis*, *H. molleri*) and in Central African (*H. cinnamomeoventris*). Sampling localities are scaled according to sample size and colored according to the mitochondrial haplotype groups represented in the population (*H. thomensis*, *H. molleri* São Tomé, or *H. molleri* Príncipe). Parsimony networks of *16s* and *cytochrome b* mitochondrial haplotypes are scaled according to sample size and colors correspond to the three main haplotype groups (*H. thomensis*, *H. molleri* São Tomé, or *H. molleri* Príncipe). Mitochondrial haplotype group and individual assignment probabilities from the STRUCTURE analysis of 3857 SNP genotypes are depicted for K=3. (Airport Army Depot), AB (Abade), BA (Baie das Agulahs), BB (Road to Bom Bom), BM (Bem Posta), BS (Bom Sucesso), CA (Caxueira), CD (Chada Água Doutor), CG (Caxão Grande), CO (Conceição), JA (Java), LA (Lagoa Amélia), MC (Monte Café), PM (Praia Melão), PP (Pico de Príncipe), PR (Papagaio River), QI (Quisinda), RT (Radio Tower), SA (Santy), SU (Road to Sundy), TB (Terra Batata)

female SVL > 40 mm; *H. molleri* male SVL < 30 mm female SVL < 33 mm; (Schiøtz 1999)]. Tissue samples (liver) were preserved in 95% ethanol or RNAlater for subsequent DNA extraction and genetic analyses. Preserved specimens are accessioned in the Cornell University Museum of Vertebrates (CUMV) and the California Academy of Sciences (CAS; Table 3.S1).

3.2.2 Mitochondrial diversity and divergence

We extracted total genomic DNA using a DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA) and used polymerase chain reaction (PCR) to amplify and sequence two mitochondrial fragments for each individual (*cytochrome-b* and *16s*) using published primers (Table 3.S2). PCRs were carried out in a final volume of 20μL containing: 20 ng template DNA, 1× Buffer, 0.2 μM of each primer, 0.4 mM dNTP mix, and 0.125 units of *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN, USA). Amplification was carried out with an initial denaturation for 5 min at 94 °C, followed by 35 cycles (60 s denaturation at 94 °C, 60 s annealing at 42-50°C (Table 3.S2), 60 s extension at 72 °C), and a final extension at 72 °C for 5 min. PCR products were visualized on an agarose gel, purified using ExoSAP-IT (USB Corp., Cleveland, OH, USA), and sequenced using a BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI Automated 3730xl Genetic Analyzer (Applied Biosystems). DNA sequences were edited using SEQUENCHER 5.0.1 (Gene Codes Corp., Ann Arbor, MI, USA).

Sequences were aligned using CLUSTAL X v 2.0.10 (Larkin *et al*. 2007) and we used TCS v 1.21 (Clement *et al*. 2000) to create haplotype networks for each locus. We used ARLEQUIN v 3.1 (Excoffier *et al*. 2005) to calculate nucleotide diversity based on the number of segregating sites (θs) and based on pairwise sequence comparisons ($\theta \pi$), uncorrected and net sequence divergence (*Dxy* and *Da* using the Tamura–Nei model; Tamura & Nei 1993), and F_{ST} for the three island lineages (*H. thomensis*, *H. molleri* from São Tomé, and *H. molleri* from Príncipe).

3.2.3 Single nucleotide polymorphism dataset collection

We used the double-digest RADseq laboratory protocol (ddRADseq; Peterson *et al*. 2012) to collect genome-wide single nucleotide polymorphism (SNP) data from a representative subset of *H. molleri* (17 from Príncipe and 54 from São Tomé) and *H. thomensis* (seven from São Tomé) as well as the six samples of *H. cinnamomeoventris* from continental Central Africa (Figure 1). For each sample we digested 1000 ng of freshly extracted DNA with the restriction enzymes S*bf1* and *MspI* (New England Biolabs, Ipswich, Massachusetts), which have 8 bp (5□-CCTGCAGG-3□) and 4 bp (5□-CCGG-3□) recognition sites, respectively. DNA digests were purified with Agencourt AMPure beads prior to ligating barcoded Illumina adaptors to the fragments. We pooled equimolar quantities of each sample prior to size selection using a Blue Pippin Prep (fragment size range 430 – 530) and PCR-amplified the libraries with 12 cycles using proofreading *Taq* and Illumina's indexed primers (all of which differed by at least two base pairs to reduce de-multiplexing errors). To check the quality of our libraries we quantified the concentration of the pooled samples using

Qubit Fluorometric Quantitation (Invitrogen, Carlsbad, California) and confirmed the fragment sizes in our libraries on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, California). We sequenced two pooled libraries of 44 samples each on two lanes of Illumina HiSeq 2000 (100-bp, single end) at the Cornell University Genomics Facility.

We processed Illumina data with the STACKS pipeline v 1.13 (Catchen *et al*. 2011, 2013), which identifies putative loci and infers haplotypes for each individual. To create putative loci and detect SNPs at each locus we implemented the ustacks program, which uses a maximum likelihood framework to group reads into loci that differ by a threshold of two mismatches with a minimum depth of coverage of five reads. Using cstacks, we generated a catalogue of consensus loci by merging unique loci across all individuals with a mismatch threshold of two differences allowed between sample tags. Finally, we resolved haplotypes for each individual for each locus in the catalogue using sstacks. To check for consistency of results between library preparations we replicated two samples (*H. thomensis* CAS251635 and *H. molleri* CAS233703) in each library and processed the reads through the STACKS pipeline as described above. We assessed repeatability of SNP calls for each sample by comparing haplotype assignments for loci recovered in both of the replicated library preparations.

Mitochondrial and nuclear loci differ in their patterns of inheritance and effective population sizes; therefore, inferences of population genetic structure, historical population demography, and gene flow based on these two classes of loci are not

always concordant (Birky *et al*. 1989, Ballard & Whitlock 2004). RADseq methods generate SNP data for both types of markers, therefore, to differentiate between mitochondrial versus nuclear SNPs in our dataset we Blasted all loci recovered in STACKS to the NCBI Vertebrate Nucleotide Database (www.blast.ncbi.nlm.nih.gov) and removed all loci that mapped to mitochondrial sequences from subsequent analyses. To generate output files for downstream analyses we used the STACKS program populations and modified the files using custom perl scripts. For analyses that included only the island taxa we included loci that were present in all three lineages and present in at least 75% of individuals in a lineage (25% missing data). For analyses that included the mainland taxon we included a representative subset of island samples (five for each lineage) and only included loci that were present in at least two individuals within each lineage (60% missing data).

3.2.4 Population structure and phylogenetic relationships of island endemics

We used the program STRUCTURE v 2.3.4 (Pritchard *et al*. 2000) to determine the number of genetic demes and degree of admixture among demes present in our samples of *H. molleri* and *H. thomensis* from São Tomé and Príncipe. We used 3857 SNPs from our RADseq dataset (we filtered data to include only one SNP per RAD locus), implemented the admixture model with correlated allele frequencies among populations and performed 10 runs at each value of *K* (ranging from one to four), with a burn-in of 1,000,000 steps and MCMC length of 5,000,000 steps. We plotted log-likelihood scores for the range of *K*-values (Evanno *et al*. 2005) to determine the most likely number of genetic clusters in the dataset and used STRUCTURE HARVESTER (Earl

& vonHoldt 2011) to combine individual assignment probabilities across replicate runs.

To estimate a species tree from the subset of SNPs represented in the three island lineages and the mainland sister taxon (n=467), we used the Bayesian program SNAPP v 1.1.1 (Bryant *et al*. 2012). SNAPP estimates the species tree from unlinked bi-allelic SNPs and makes the assumption of no gene flow between lineages; therefore, we filtered our dataset to include a single bi-allelic SNP from each RAD locus present in all four lineages and selected individuals of *H. molleri* (five each from São Tomé and Príncipe) and *H. thomensis* (five from São Tomé) with no evidence of admixed ancestry in the STRUCTURE analysis. We used BEAUti to generate the input file with default settings for SNAPP, ran the analysis for two replicate runs of 5,000,000 MCMC steps, and assessed convergence using TRACER (Rambaut *et al*. 2013). The effective sample size for all parameters was well above 200 and we discarded the first 10% of trees as burn-in prior to summarizing the distribution of topologies in the dataset with TREESETANALYZER. We visualized the distribution of species tree topologies and node heights using DENSITREE (Bouckaert 2010).

To compare relative diversity within and divergence between the island lineages, we used ARLEQUIN v 3.1 (Excoffier *et al*. 2005) to calculate F_{ST}, the proportion of polymorphic sites (P), theta based on expected homozygosity (θ_H; Zouros 1979, Chakraborty & Weiss 1991), and expected heterozygosity (H_E) versus observed heterozygosity (H_O) on the set of SNPs used in the STRUCTURE analysis (n=3857). We

measured mean allelic richness (N_A) with HP-RARE v 1.0 (Kalinowski 2005), which uses rarefaction and hierarchical sampling to adjust for uneven sample sizes across localities.

3.2.5 Identification and classification of hybrids

To quantify the extent of potential hybridization between *H. molleri* and *H. thomensis* on São Tomé, we used NEWHYBRIDS (Anderson & Thompson 2002) to compute the posterior probability that an individual belongs to distinct genotype frequency classes (parental, F1, F2, and backcrosses). We used 386 SNPs from our RADseq dataset (we filtered data to include only one SNP per RAD locus and SNPs with a minor allele frequency > 0.2) and performed four replicate runs of 1,000,000 sweeps and a burn-in of 100,000 sweeps with default genotype categories. For individuals with assignment probabilities > 0.99 to either the *H. molleri* or *H. thomensis* demes in the STRUCTURE analysis, we specified the corresponding genotype frequency class (parental *H. molleri* or *H. thomensis*) using the z option in the input data file. To account for the potential influence of priors on hybrid classification we performed two runs with uniform priors and two runs with Jeffrey's priors for the mixing proportions and allele frequencies. We assessed convergence by comparing *P*(z) values from the replicate runs.

3.3 Results

3.3.1 Mitochondrial diversity and divergence

We recovered three differentiated mitochondrial haplotype groups that correspond to *H. thomensis*, the São Tomé lineage of *H. molleri*, and the Príncipe lineage of *H.*

molleri (Figure 3.1). Although populations of *H. molleri* on both islands are currently considered one species, they do not share any mitochondrial haplotypes. In contrast, *H. thomensis* and *H. molleri* are considered distinct species yet seven *H. molleri* from Lagoa Amélia (LA) and Bom Sucesso (BS) on São Tomé carry *H. thomensis* mitochondrial haplotypes (Figure 3.1; Table 3.S1). The three lineages are highly differentiated from one another (Da = 1.1 – 2.7%, F_{ST} = 0.77-0.97; Table 3.1) and genetic diversity is greater within the São Tomé lineage of *H. molleri* than the Príncipe lineage (Table 3.2).

3.3.2 Single nucleotide polymorphism dataset

We generated approximately 200 million sequence reads after filtering raw reads for quality, intact restriction sites, and matches to sample barcodes (average of ~2.4 million reads per sample). The STACKS pipeline generated an average of ~28,000 unique loci per sample with an average depth of coverage of 68X per SNP. The replicated samples (*H. thomensis* CAS251635 and *H. molleri* CAS233703) indicate that the ddRADseq protocol is reasonably repeatable with shared haplotype calls recovered for 91.2% of 4579 and 93.4% of 6050 loci shared across replicate runs, respectively. Discrepancies between replicate runs are mainly attributable to a heterozygous versus a homozygous call for an individual (6.1-7.5% of loci) and the frequency of entirely conflicting calls between replicates was very low (0.5%-1.4% of loci). Six loci in the STACKS catalog that matched mitochondrial genes in the BLAST search were excluded from subsequent analyses.

Table 3.1 Estimates of pairwise F_{ST} values between *Hyperolius molleri* (São Tomé and Príncipe Islands), *H. thomensis* (São Tomé Island), and *H. cinnamomeoventris* (Gabon) for mtDNA (*cytochrome-b/16s)* and nuDNA (3857 RADseq SNPs). *H. cinnamomeoventris* are not included for nuDNA comparisons because a small subset of RADseq loci were shared across all four taxa. All values are significant at $p < 0.001$.

	H. cinn	*H. moll* Príncipe	*H. moll* São Tomé
H. moll Príncipe (mtDNA)	0.84/0.85	--	
H. moll Príncipe (nuDNA)	--	--	
H. moll São Tomé (mtDNA)	0.87/0.90	0.79/0.86	--
H. moll São Tomé (nuDNA)	--	0.489	--
H. thom São Tomé (mtDNA)	0.77/0.84	0.97/0.96	0.82/0.85
H. thom São Tomé (nuDNA)	--	0.696	0.400

Table 3.2 Summary statistics for mitochondrial loci and nuclear SNPs collected from *Hyperolius molleri* (São Tomé and Príncipe Islands), *H. thomensis* (São Tomé Island), and *H. cinnamomeoventris* (Gabon, Central Africa).

	16s					*Cytochrome b*					*Nuclear SNPs*						
Lineage	N	bp	N_h	θs	θπ	N	bp	N_h	θs	θπ	N	Sites	P	N_A	H_O	H_E	θ_H
H. cinn	5	523	3	0.0358	0.0478	6	616	5	0.0647	0.0902	--	--	--	--	--	--	--
H. moll Príncipe	21	521	1	0.0000	0.0000	22	616	3	0.0009	0.0003	17	3857	0.23	1.15	0.05	0.05	0.052
H. moll São Tomé	68	521	7	0.0038	0.0026	57	616	8	0.0077	0.0055	54	3857	0.72	1.33	0.09	0.11	0.118
H. thom São Tomé	20	521	4	0.0022	0.0011	14	616	5	0.0031	0.0018	7	3857	0.32	1.23	0.08	0.09	0.084

N, number of individuals sampled, bp, sequence length in base pairs; N_h, number of haplotypes; θs, genetic diversity based on the number of segregating sites; θπ, genetic diversity based on pairwise sequence comparisons. P, proportion of polymorphic sites, N_A, allelic richness corrected for uneven sample size, H_O, observed heterozygosity, H_E, expected heterozygosity, θ_H, genetic diversity based on expected homozygosity.

3.3.3 Population structure and phylogenetic relationships of island endemics

Our STRUCTURE analysis of 3857 SNPs for the island samples recovered three demes corresponding to *H. thomensis*, the São Tomé lineage of *H. molleri*, and the Príncipe lineage of *H. molleri*. Consistent with the mitochondrial haplotype networks, we find no evidence of admixture between the São Tomé and Príncipe lineages of *H. molleri* (Figure 3.1). Also consistent with the mtDNA data, several *H. molleri* from sites between 1000 and 1400 m on São Tomé (Lagoa Amélia, Terra Batata, and Bom Sucesso) exhibit substantial admixture with the *H. thomensis* deme (Figure 3.1). Despite extensive admixture between *H. molleri* and *H. thomensis* on São Tomé, we recovered considerable genetic differentiation among all three island lineages in our SNP dataset (F_{ST} = 0.400-0.696; Table 3.1). Estimates of heterozygosity, allelic richness, and the proportion of polymorphic sites indicate that genetic diversity is greater within the São Tomé lineage of *H. molleri* than the Príncipe lineage (Table 3.2).

Divergence at mitochondrial loci between mainland *H. cinnamomeoventris* and the island species ranged from 3.7-4.5% for *16s* and 7.7-8.5% for *cytochrome b*, consequently we recovered fewer shared bi-allelic SNPs across these more divergent lineages (n=467). Our SNAPP species tree analysis confirms that the island lineages form a monophyletic group that is well differentiated from the mainland sister taxon (Figure 3.2). Consistent with the current taxonomy, we recovered a sister relationship between *H. molleri* populations from Príncipe and São Tomé, and monophyly of *H.*

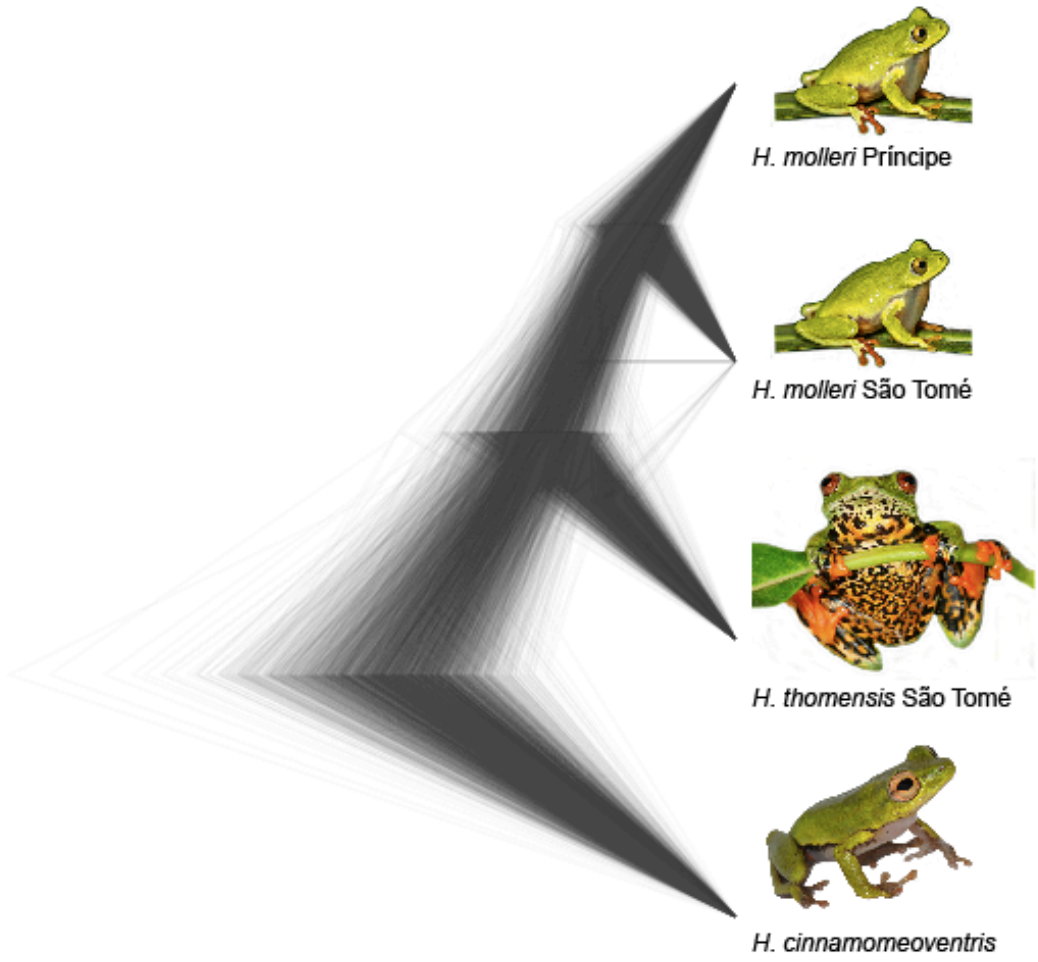

Figure 3.2 SNAPP species tree inferred from 467 nuclear bi-allelic SNPs shared among *Hyperolius thomensis* (5), *H. molleri* from São Tomé (5), *H. molleri* from Príncipe (5) and the *H. cinnamomeoventris* (6) complex from Gabon. Branch lengths are a relative measure of substitutions per site. All nodes are supported by posterior probabilities greater than 0.99. Photo credits A. Stanbridge, D. Lin, B. Stuart.

molleri relative to *H. thomensis* (Figure 3.2). All nodes in the phylogeny are well supported (posterior probability = 0.99).

3.3.5 Identification and classification of hybrids

Using the subset of 386 SNPs with a minor allele frequency > 0.2, NEWHYBRIDS identified 17 individuals of *H. molleri* as hybrids with posterior probability > 0.99 (six F2 hybrids, nine *H. molleri* backcross hybrids, and two *H. thomensis* backcross hybrids; Figure 3.3). The majority of these hybrids are from three sites where the ranges of *H. thomensis* and *H. molleri* are sympatric on São Tomé (Lagoa Amélia, Bom Sucesso, and Terra Batata; Figure 3.3). Of the seven *H. molleri* that carry *H. thomensis* mitochondrial haplotypes, one was classified by NEWHYBRIDS as *H. thomensis* backcross, five as *H. molleri* backcross, and one as *H. molleri* parental (Figure 3.3). The individual classified as *H. molleri* parental is a male we collected in an agricultural field (Bom Sucesso) that was assigned to the *H. molleri* deme in the STRUCTURE analysis with $Q = 0.92$. Given the mixed mitochondrial and nuclear ancestry of this male we consider that mixed ancestry in this individual likely results from multiple generations of backcrossing with *H. molleri*.

3.4 Discussion

3.4.1 Dispersal and colonization in the Gulf of Guinea does not follow the progression rule

Although the island of São Tomé is much younger than Príncipe (13 Myr versus 30 Myr) our species tree indicates that *Hyperolius* initially colonized São Tomé and

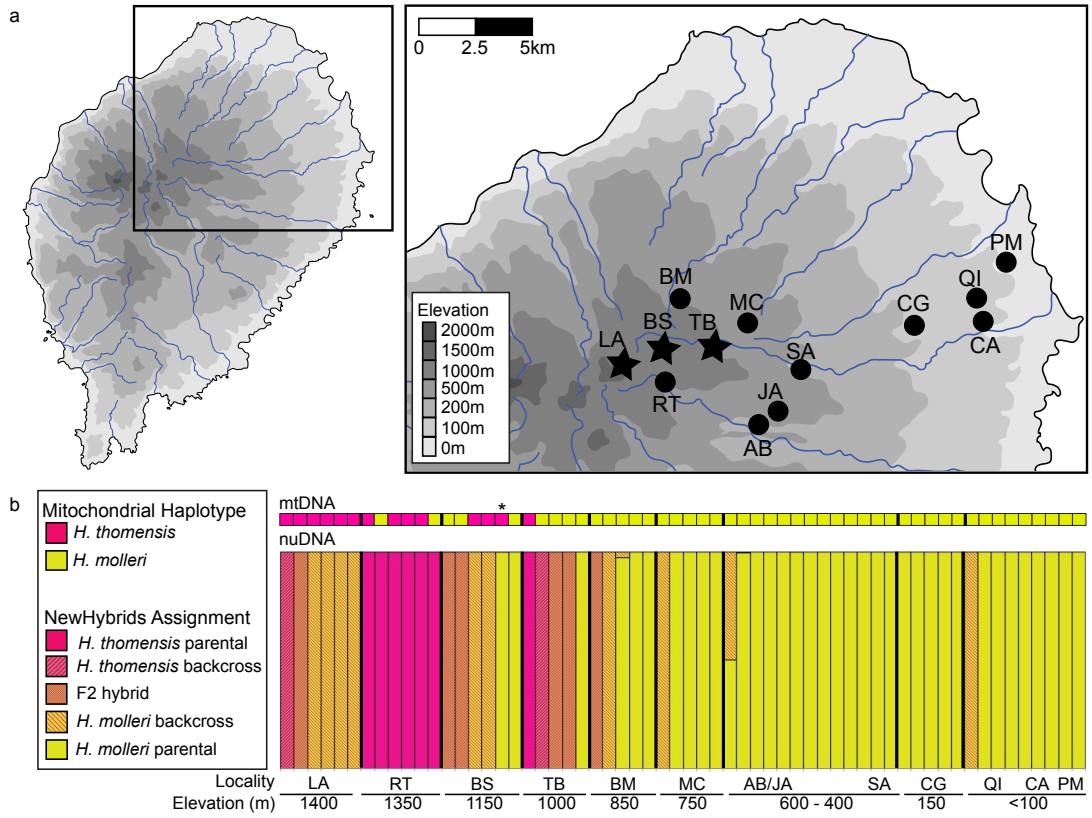

Figure 3.3 (a) Sampling localities on the island of São Tomé; stars denote localities with high proportions of F2 and backcross hybrid individuals. (b) *Hyperolius thomensis* and *H. molleri* mitochondrial haplotype group and hybrid classification from the NEWHYBRIDS analysis of 386 SNP genotypes. The asterisk denotes an individual classified by NEWHYBRIDS as *H. molleri* parental that carries an *H. thomensis* mitochondrial haplotype. AB (Abade), BM (Bem Posta), BS (Bom Sucesso), CA (Caxueira), CG (Caxão Grande), JA (Java), LA (Lagoa Amélia), MC (Monte Café), PM (Praia Melão), QI (Quisinda), RT (Radio Tower), SA (Santy), TB (Terra Batata).

subsequently dispersed to Príncipe. Estimates of genetic diversity (e.g. number of polymorphic sites, θ_H, and allelic richness; Table 3.2) of *H. molleri* on Príncipe are much lower than for São Tomé populations, which is consistent with this colonization order. Most instances of inter-island dispersal in well-studied island systems such as the Canary, Hawaiian, and Galapagos archipelagos follow the progression rule (Wagner & Funk 1995) and proceed from older to younger islands (Juan *et al*. 2000, Cowie & Holland 2008, Parent *et al*. 2008). The biased direction of colonization is often attributed to the greater availability of ecological niche space on younger islands (Gillespie & Roderick 2002). Exceptions to this pattern indicate that other physical attributes of islands, including wind patterns, ocean currents, and migration routes, also shape overall patterns of inter-island dispersal (Cowie & Holland 2006). For *Hyperolius* and other organisms that rely on rafting to disperse overseas, we expect that dispersal between islands is largely determined by ocean currents, which flow from south to north (Annobón to São Tomé to Príncipe) in the Gulf of Guinea. Few phylogenetic studies are available for such taxa in the Gulf of Guinea, but mitochondrial studies of island *Afroablepharus* skinks and *Lygodactylus* geckos are consistent with a south to north dispersal pattern (Jesus *et al*. 2006, 2007).

3.4.2 Inter-island dispersal and allopatric divergence in H. molleri

Hyperolius molleri populations on São Tomé and Príncipe are strongly differentiated at mtDNA and nuDNA, which confirms that dispersal between the islands is possible for *Hyperolius* but is not ongoing. Although the islands are only separated by approximately 150 km, none of the six other endemic amphibians that occur on

Príncipe or São Tomé have successfully dispersed between the islands, further indicating that such dispersal events are uncommon for amphibians. We previously estimated divergence between populations of *H. molleri* on the two islands at approximately 1.1 Myr to 270 kyr (Bell *et al. in review*), indicating that *H. molleri* colonized Príncipe very recently in the island's 30 Myr evolutionary history. Successful dispersal and recruitment on older islands is typically limited by the availability of ecological niches (Gillespie & Roderick 2002), but Príncipe only hosts two other amphibian species (a large-bodied treefrog, *Leptopelis palmatus* and a leaf litter species, *Phrynobatrachus dispar*) that are unlikely to compete with *H. molleri*. Therefore, though *in situ* diversification eventually eclipses dispersal in the accumulation of biodiversity on older islands (Emerson & Oromí 2005), dispersal may continue to play an important role for groups that rarely disperse overseas and remain relatively depauperate on oceanic islands.

Populations of *H. molleri* on the two islands are currently considered a single species because they are phenotypically similar and occupy similar habitats (Drewes & Stoelting 2004). Our study clearly indicates that they represent evolutionarily distinct lineages, however, as they do not share mtDNA haplotypes and form entirely distinct genetic demes in our STRUCTURE analysis of genome wide SNPs (Figure 3.1). The consistency of these results despite fairly recent population divergence, as well as lower genetic diversity in Príncipe *H. molleri* compared to São Tomé populations (Table 3.2), indicates that founder effects and genetic drift have likely augmented genetic differentiation between the two lineages. These micro-evolutionary processes

result in large shifts in allele frequencies (Nei *et al*. 1975, Dlugosch & Parker 2008) and accelerate rates of lineage sorting (Kimura & Ohta 1969), which can generate phenotypic divergence over short evolutionary timescales when coupled with divergent ecological selection (Velo-Anton *et al*. 2011). The absence of phenotypic differentiation between the island populations of *H. molleri* may therefore indicate that the selective environments on São Tomé and Príncipe are similar. Alternatively, closer examination of the morphology and ecology of *H. molleri* on the two islands may reveal previously unrecognized phenotypic differentiation between these genetically diverged lineages.

3.4.3 Breeding site availability and divergence in allopatry drive in situ diversification on São Tomé

Our species tree analysis confirms that divergence between *H. molleri* and *H. thomensis* occurred *in situ* on the island of São Tomé and we recover substantial admixture between the species where their ranges are sympatric, which is consistent with our observations of individuals with intermediate phenotypes at these sites (Bell & Drewes, unpublished data). Allopatric divergence can produce patterns of genetic admixture either due to incomplete lineage sorting or secondary introgression (Maddison 1997), however, the geographic pattern of divergence we recover (admixture decreases with increasing distance from the zone of sympatry) is more consistent with allopatric speciation and secondary contact than with incomplete lineage sorting. Despite relatively recent divergence between the two species (1.7 to 0.5 Myr; Bell *et al*. *in review*), *H. thomensis* is 50% larger than *H. molleri* and breeds

exclusively in water-filled tree cavities (Drewes & Stoelting 2004), implicating a role for divergent ecological selection in driving divergence between the species.

We propose that geographic segregation in the availability and type of breeding habitats on São Tomé may have driven initial allopatric divergence between *H. molleri* and *H. thomensis*. *Hyperolius molleri* breed along slow moving streams and water-filled ditches, which are typical breeding sites for *Hyperolius* species, including the mainland sister taxon *H. cinnamomeoventris* (Schiøtz 1999). The absence of small streams at higher elevations on São Tomé may underlie the evolution of tree cavity (phytotelm) breeding in *H. thomensis* although this specialized reproductive mode is typically associated with avoiding predation and competition encountered in stream or pond habitats (Lehtinen *et al*. 2004). This same mechanism may also explain what has brought these previously allopatric lineages into secondary contact. The hybrid zone, which extends from approximately 1000 to 1400 m elevation on Pico de São Tomé, coincides with a transition from agricultural land to primary forest. Most of the *H. molleri* breeding sites at these elevations are artificial and associated with agriculture (e.g. cisterns); thus the expansion of agriculture may have increased the availability of *H. molleri* breeding sites at higher elevations. This region is also coincident with the well-studied *Drosophila santomea*/*D. yakuba* hybrid zone (Lachaise *et al*. 2000, Llopart *et al*. 2005, Matute *et al*. 2009). Like *Hyperolius*, the two species of *Drosophila* are ecologically isolated and differences in habitat and temperature preference contribute to both pre-mating and post-mating reproductive barriers in these species (Matute *et al*. 2009, Matute & Coyne 2010). Therefore the expansion of

agriculture at mid to high elevations on São Tomé may have promoted secondary contact and hybridization in both *Drosophila* and *Hyperolius*. Sympatry and the potential for hybridization between *H. molleri* and *H. thomensis* may predate agricultural development on São Tomé, however, because one of our sample sites is a natural crater lake (Lagoa Amélia) at approximately 1400 m elevation on the Pico de São Tomé that hosts a large breeding population of *H. molleri*.

Hybridization between *H. molleri* and *H. thomensis* is very common at the sympatric sites we sampled but the extent of introgression is geographically constrained; we do not find *H. thomensis* mitochondrial haplotypes beyond Bom Sucesso and the proportion of individuals classified as hybrids (F2 or backcross) in the NEWHYBRIDS analysis drops precipitously where the species are allopatric. These patterns may reflect selection against hybrids (Barton & Hewitt 1985); however, strong selection against hybridization seems unlikely as the 17 hybrids we sampled were breeding adults that were classified as F2 and backcross hybrids, indicating that hybrid progeny are likely viable and fertile (Coyne & Orr 1998). Therefore it may be that a difference in preferred breeding sites is the primary reproductive barrier for these two species.

Although our sampling of *H. thomensis* is limited (20 individuals from two sites) we did not find any *H. thomensis* carrying *H. molleri* mitochondrial haplotypes indicating that hybridization may be asymmetrical. This apparent asymmetry in hybridization may result from sexual differences in dispersal and mating behavior (Lamb & Avise 1986, Cahill *et al*. 2013) such that male *H. thomensis* breed exclusively in tree cavities

while female *H. thomensis* visit both *H. molleri* and *H. thomensis* breeding sites. The male advertisement calls of the two species are not dramatically different and we collected a female *H. thomensis* in amplexus with a male *H. molleri* in a cistern at Terra Batata (an agricultural field at 1000m; Figure 3.1); therefore, it is feasible that artificial breeding sites between 1000 and 1400 m and sexual differences in mating behavior have facilitated asymmetrical hybridization between these species. Alternatively, the absence of *H. thomensis* carrying *H. molleri* mitochondrial haplotypes and higher prevalence of *H. molleri* backcross hybrids relative to *H. thomensis* backcross hybrids may indicate strong selection against progeny from *H. thomensis* male and *H. molleri* female matings (Coyne & Orr 1998).

In summary, our results indicate that initial population divergence between *H. molleri* and *H. thomensis* on São Tomé was likely allopatric due to the geographic separation of available breeding sites and that secondary contact has resulted in hybridization and extensive introgression between the species. The evolution of gigantism and a specialized reproductive mode in *H. thomensis* despite fairly recent divergence between *H. molleri* and *H. thomensis* (1.7 to 0.5 Mya; Bell *et al. in review*) highlight a role for divergent ecological or sexual selection in driving rapid phenotypic differentiation between the species. Future studies quantifying selection on these phenotypes across the hybrid zone may identify the selective pressures that initially drove divergence between *H. molleri* and *H. thomensis* and highlight mechanisms that underlie the evolution of gigantism on islands (Lomolino 1985) and the evolution of phytotelm breeding in frogs (Lehtinen *et al.* 2004).

3.5 Acknowledgments

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SUPPLEMENTAL INFORMATION FOR CHAPTER 3 - EVOLUTIONARY GENOMICS OF DIVERSIFICATION IN THE GULF OF GUINEA: DISPERSAL AND IN SITU SPECIATION IN AFRICAN REED FROGS

Table 3.S1 Sampling localities and voucher information. Hybrid individuals as identified by mitochondrial haplotypes or NewHybrid classifications assignments (P (z) > 0.95) are shown in bold. Abbreviations as follow: GAOI (Gabon-Ogooué-Ivindo), GAOM (Gabon-Ogooué-Maritime), PRAA (Príncipe-Airport Army Depot), PRBA (Príncipe-Baie das Agulahs), PRBB (Príncipe-Road to Bom Bom), PRCD (Príncipe-Chada Água Doutor), PRCO (Príncipe-Conceição), PRPP (Príncipe-Pico de Príncipe), PRPR (Príncipe-Papagaio River), PRSU (Príncipe-Road to Sundy), STAB (São Tomé-Abade), STBM (São Tomé-Bem Posta), STBS (São Tomé-Bom Sucesso), STCA (São Tomé-Caxueira), STCG (São Tomé-Caxão Grande), STJA (São Tomé-Java), STLA (São Tomé-Lagoa Amélia), STMC (São Tomé-Monte Café), STPM (São Tomé-Praia Melão), STQI (São Tomé-Quisinda), STRT (São Tomé-Radio Tower), STSA (São Tomé-Santy), STTB (São Tomé-Terra Batata).

Species	Locality	Catalog No.	Lat	Long	mtDNA Haplotype	NewHybrids Assignment
H. cinn	GAOI	CU15026	0.5112	12.8028	--	--
H. cinn	GAOI	CU15067	0.5112	12.8028	--	--
H. cinn	GAOI	CU15496	-0.2095	12.2905	--	--
H. cinn	GAOI	CU15514	-0.2095	12.2889	--	--
H. cinn	GAOM	CU15092	-1.8140	9.3556	--	--
H. cinn	GAOM	CU15105	-1.8914	9.5682	--	--
H. moll	PRAA	CAS219148	1.6685	7.4128	*H. moll* PR	--
H. moll	PRBA	CAS219203	1.6009	7.3531	*H. moll* PR	--
H. moll	PRBB	CAS238886	1.6892	7.4027	*H. moll* PR	--
H. moll	PRBB	CAS238887	1.6892	7.4027	*H. moll* PR	--
H. moll	PRBB	CAS238888	1.6892	7.4027	*H. moll* PR	--
H. moll	PRBB	CAS238889	1.6892	7.4027	*H. moll* PR	--
H. moll	PRBB	CAS238890	1.6892	7.4027	*H. moll* PR	--
H. moll	PRBB	CAS253047	1.6883	7.4022	*H. moll* PR	--
H. moll	PRBB	CAS253048	1.6883	7.4022	*H. moll* PR	--
H. moll	PRBB	CAS253049	1.6883	7.4022	*H. moll* PR	--
H. moll	PRBB	CAS253050	1.6883	7.4022	*H. moll* PR	--
H. moll	PRBB	CAS253051	1.6883	7.4022	*H. moll* PR	--
H. moll	PRCD	CAS219125	1.6521	7.4161	*H. moll* PR	--
H. moll	PRCD	CAS219126	1.6521	7.4161	*H. moll* PR	--
H. moll	PRCD	CAS219128	1.6521	7.4161	*H. moll* PR	--

Species	Locality	Catalog No.	Lat	Long	mtDNA Haplotype	NewHybrids Assignment
H. moll	PRCD	CAS219129	1.6521	7.4161	*H. moll* PR	--
H. moll	PRCO	CAS219192	1.6441	7.3978	*H. moll* PR	--
H. moll	PRPP	CAS233444	1.5881	7.3808	*H. moll* PR	--
H. moll	PRPR	CAS233491	1.6259	7.4166	*H. moll* PR	--
H. moll	PRPR	CAS233492	1.6259	7.4166	*H. moll* PR	--
H. moll	PRPR	CAS233493	1.6259	7.4166	*H. moll* PR	--
H. moll	PRPR	CAS233494	1.6259	7.4166	*H. moll* PR	--
H. moll	PRSU	CAS233422	1.6611	7.3941	*H. moll* PR	--
H. moll	STAB	CAS233703	0.2541	6.6446	*H. moll* ST	--
H. moll	STBM	CAS251583	0.3082	6.6167	*H. moll* ST	--
H. moll	STBM	CAS251584	0.3082	6.6167	*H. moll* ST	--
H. moll	STBM	CAS251585	0.3082	6.6167	*H. moll* ST	F2 hybrid
H. moll	STBM	CAS251586	0.3082	6.6167	*H. moll* ST	*H. molleri*
H. moll	STBM	CAS251587	0.3082	6.6167	*H. moll* ST	HM backcross
H. moll	STBS	CAS219054	0.2885	6.6031	*H. moll* ST	--
H. moll	STBS	CAS219055	0.2885	6.6031	*H. moll* ST	--
H. moll	STBS	CAS233463	0.2887	6.6125	***H. thom***	HM backcross
H. moll	STBS	CAS233464	0.2887	6.6125	*H. moll* ST	--
H. moll	STBS	CAS233465	0.2887	6.6125	***H. thom***	HM backcross
H. moll	STBS	CAS233466	0.2887	6.6125	*H. moll* ST	--
H. moll	STBS	CAS233467	0.2887	6.6125	*H. moll* ST	--
H. moll	STBS	CAS251593	0.2882	6.6131	*H. moll* ST	F2 hybrid
H. moll	STBS	CAS251594	0.2882	6.6131	***H. thom***	*H. molleri*
H. moll	STBS	CAS251595	0.2882	6.6131	*H. moll* ST	F2 hybrid
H. moll	STBS	CAS251596	0.2882	6.6131	*H. moll* ST	*H. molleri*
H. moll	STCA	CAS218848	0.2980	6.7304	*H. moll* ST	--
H. moll	STCA	CAS218849	0.2980	6.7304	*H. moll* ST	--
H. moll	STCA	CAS218850	0.2980	6.7304	*H. moll* ST	--
H. moll	STCA	CAS218852	0.2980	6.7304	*H. moll* ST	--
H. moll	STCA	CAS218861	0.2980	6.7304	*H. moll* ST	--
H. moll	STCA	CAS218862	0.2980	6.7304	*H. moll* ST	--
H. moll	STCA	CAS218863	0.2980	6.7304	*H. moll* ST	--
H. moll	STCA	CAS218864	0.2980	6.7304	*H. moll* ST	--
H. moll	STCA	CAS218865	0.2980	6.7304	*H. moll* ST	--
H. moll	STCA	CAS251622	0.3023	6.7323	*H. moll* ST	*H. molleri*
H. moll	STCA	CAS251623	0.3023	6.7323	*H. moll* ST	*H. molleri*
H. moll	STCA	CAS251624	0.3023	6.7323	*H. moll* ST	*H. molleri*
H. moll	STCA	CAS251625	0.3023	6.7323	*H. moll* ST	*H. molleri*
H. moll	STCA	CAS251626	0.3023	6.7323	*H. moll* ST	*H. molleri*

Species	Locality	Catalog No.	Lat	Long	mtDNA Haplotype	NewHybrids Assignment
H. moll	STCG	CAS253070	0.2969	6.7038	*H. moll* ST	*H. molleri*
H. moll	STCG	CAS253071	0.2969	6.7038	*H. moll* ST	*H. molleri*
H. moll	STCG	CAS253072	0.2969	6.7038	*H. moll* ST	*H. molleri*
H. moll	STCG	CAS253073	0.2969	6.7038	*H. moll* ST	*H. molleri*
H. moll	STCG	CAS253074	0.2969	6.7038	*H. moll* ST	*H. molleri*
H. moll	STJA	CAS218974	0.2611	6.6509	*H. moll* ST	--
H. moll	STJA	CAS218975	0.2611	6.6509	*H. moll* ST	--
H. moll	STJA	CAS218986	0.2611	6.6509	*H. moll* ST	--
H. moll	STJA	CAS218987	0.2611	6.6509	*H. moll* ST	--
H. moll	STJA	CAS218988	0.2611	6.6509	*H. moll* ST	--
H. moll	STJA	CAS253055	0.2616	6.6512	*H. moll* ST	*H. molleri*
H. moll	STJA	CAS253056	0.2616	6.6512	*H. moll* ST	*H. molleri*
H. moll	STJA	CAS253057	0.2616	6.6512	*H. moll* ST	*H. molleri*
H. moll	STJA	CAS253058	0.2616	6.6512	*H. moll* ST	*H. molleri*
H. moll	STJA	CAS253059	0.2616	6.6512	*H. moll* ST	*H. molleri*
H. moll	STLA	CAS219048	0.2816	6.5909	*H. moll* ST	--
H. moll	STLA	CAS219049	0.2816	6.5909	***H. thom***	HM backcross
H. moll	STLA	CAS219050	0.2816	6.5909	***H. thom***	HM backcross
H. moll	STLA	CAS219059	0.2885	6.6031	***H. thom***	HT backcross
H. moll	STLA	CAS251613	0.2815	6.5908	***H. thom***	HM backcross
H. moll	STLA	CAS251614	0.2815	6.5908	*H. moll* ST	*H. molleri*
H. moll	STLA	CAS251615	0.2815	6.5908	*H. moll* ST	F2 hybrid
H. moll	STLA	CAS251616	0.2815	6.5908	*H. moll* ST	HM backcross
H. moll	STMC	CAS251606	0.2961	6.6381	*H. moll* ST	*H. molleri*
H. moll	STMC	CAS251607	0.2961	6.6381	*H. moll* ST	HM backcross
H. moll	STMC	CAS251608	0.2961	6.6381	*H. moll* ST	*H. molleri*
H. moll	STMC	CAS251609	0.2961	6.6381	*H. moll* ST	*H. molleri*
H. moll	STMC	CAS251610	0.2961	6.6381	*H. moll* ST	*H. molleri*
H. moll	STPM	CAS219068	0.3188	6.7384	*H. moll* ST	*H. molleri*
H. moll	STQI	CAS219010	0.3011	6.7320	*H. moll* ST	*H. molleri*
H. moll	STQI	CAS219011	0.3011	6.7320	--	*H. molleri*
H. moll	STQI	CAS219047	0.3011	6.7320	*H. moll* ST	HM backcross
H. moll	STSA	CAS218839	0.2791	6.6602	*H. moll* ST	*H. molleri*
H. moll	STSA	CAS218840	0.2791	6.6602	*H. moll* ST	*H. molleri*
H. moll	STSA	CAS253064	0.2770	6.6593	*H. moll* ST	*H. molleri*
H. moll	STSA	CAS253065	0.2770	6.6593	*H. moll* ST	*H. molleri*
H. moll	STSA	CAS253066	0.2770	6.6593	*H. moll* ST	*H. molleri*
H. moll	STSA	CAS253067	0.2770	6.6593	*H. moll* ST	*H. molleri*
H. moll	STSA	CAS253068	0.2770	6.6593	*H. moll* ST	*H. molleri*

Species	Locality	Catalog No.	Lat	Long	mtDNA Haplotype	NewHybrids Assignment
H. moll	STTB	CAS251601	0.2885	6.6240	*H. moll* ST	F2 hybrid
H. moll	STTB	CAS251602	0.2885	6.6240	*H. moll* ST	*H. molleri*
H. moll	STTB	CAS251603	0.2885	6.6240	*H. moll* ST	HT backcross
H. moll	STTB	CAS251604	0.2885	6.6240	*H. moll* ST	F2 hybrid
H. thom	STRT	CAS218925	0.2761	6.6056	*H. thom*	*H. thomensis*
H. thom	STRT	CAS218926	0.2761	6.6056	*H. thom*	*H. thomensis*
H. thom	STRT	CAS218927	0.2761	6.6056	*H. thom*	*H. thomensis*
H. thom	STRT	CAS218928	0.2761	6.6056	*H. thom*	--
H. thom	STRT	CAS218929	0.2761	6.6056	*H. thom*	--
H. thom	STRT	CAS218930	0.2761	6.6056	*H. thom*	--
H. thom	STRT	CAS218934	0.2761	6.6056	*H. thom*	--
H. thom	STRT	CAS218935	0.2761	6.6056	*H. thom*	--
H. thom	STRT	CAS218936	0.2761	6.6056	*H. thom*	--
H. thom	STRT	CAS218937	0.2761	6.6056	*H. thom*	--
H. thom	STRT	CAS233470	0.2761	6.6056	*H. thom*	--
H. thom	STRT	CAS233471	0.2761	6.6056	*H. thom*	--
H. thom	STRT	CAS233472	0.2761	6.6056	*H. thom*	--
H. thom	STRT	CAS233473	0.2761	6.6056	*H. thom*	--
H. thom	STRT	CAS233474	0.2761	6.6056	*H. thom*	--
H. thom	STRT	CAS233475	0.2761	6.6056	*H. thom*	--
H. thom	STTB	CAS251605	0.2885	6.6240	*H. thom*	*H. thomensis*
H. thom	STRT	CAS251635	0.2757	6.6041	*H. thom*	*H. thomensis*
H. thom	STRT	CAS251636	0.2757	6.6041	*H. thom*	*H. thomensis*
H. thom	STRT	CAS251637	0.2757	6.6041	*H. thom*	*H. thomensis*

Table 3.S2 Primer sequences and amplification conditions for mitochondrial sequences collected from *Hyperolius molleri* (São Tomé and Príncipe Islands), *H. thomensis* (São Tomé Island), and the *H. cinnamomeoventris* species complex (Central Africa).

	Locus Length	PCR Annealing Temperature			
Primer Sequence	(bp)	*H.cinn*	*H.moll*	*H.thom*	Reference
MVZ15 5' GAA CTA ATG GCC CAC ACW WTA CG 3'	670	43*	42*	42*	(Moritz *et al*. 1992)
MVZ16 5' AAA TAG GAA RTA TCA YTC TGG TTT RAT 3'					(Moritz *et al*. 1992)
16s A-L 5' CGC CTG TTT ATC AAA AAC AT 3'	521	50	50	50†	(Palumbi *et al*. 1991)
16s B-H 5' CCC GTC TGA ACT CAG ATC ACG T 3'					(Palumbi *et al*. 1991)

* indicates 0.3 μL of additional MgCl per reaction † indicates 0.75 μL Bovine Serum Albumin per reaction

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CHAPTER 4

CLIMATIC REFUGIA AND MARINE INCURSIONS SHAPE DIVERSIFICATION IN CENTRAL AFRICAN REED FROGS

Abstract

Diversification in rainforest organisms is frequently attributed to population contraction and expansion through Pliocene-Pleistocene climatic cycles, but other factors such as ecological gradients and vicariance due to geological activity also contribute to lineage diversification. We employ a comparative phylogeographic study across three species of reed frogs that inhabit a spectrum of habitats (rainforest, bushland, and savannah) to investigate mechanisms shaping diversification in the Guineo-Congolian forest of Central Africa. Two of our three focal species are also distributed on the land-bridge island Bioko, and we quantify the effects of marine incursions on divergence between island and mainland populations. We recover substantial phylogeographic structure across all three species although the oldest divergences in each species differ, dating to the Late Miocene or Pliocene-Pleistocene. For the two species that inhabit forest and bushland habitats, timing and degree of phylogeographic isolation correspond to differences in the species' reliance on rainforest habitats. For the third species, which inhabits bushland and savannah habitats, we find evidence for the ecotone model of speciation with aridification in the Late Miocene driving divergence into savannah and bushland lineages. Across all three species we recover a shared pattern of divergence between clades East and West

of the Congo Basin dating to the Pliocene-Pleistocene transition, underscoring the importance of genetic exchange and vicariance across the northern Congo Basin in shaping regional diversity. Patterns of divergence in the Bioko Island populations indicate that marine incursions are not the only factor restricting dispersal between Bioko and the continent. Instead, the composition of habitats connecting Bioko to the rest of the continent when sea-levels retreat may restrict dispersal across the land-bridge in some species and not in others. The spectrum of divergence times across our three focal taxa, as well as regions of secondary contact between previously isolated lineages, present a rich comparative framework in which to investigate the accumulation of reproductive isolation and phenotypic divergence in Central African vertebrates.

4.1 Introduction

Diversification in rainforest organisms is frequently attributed to population contraction and expansion through Pliocene-Pleistocene climatic cycles during the past several million years (Haffer 1969). These climatic oscillations shaped the global distribution of biomes and sea levels, with concomitant effects on the distributions and diversification of species. The role of these climatic fluctuations in lineage diversification is well documented for organisms inhabiting montane tropical forests in the Brazilian Atlantic Forest and the Australia Wet Tropics Rainforest (e.g. Hugall *et al*. 2002, Carnaval *et al*. 2009, Bell *et al*. 2010, Singhal & Moritz 2013). Studies in the East Afromontane Forests of Central Africa, however, find mixed support for the

influence of climate shifts on lineage diversification (Couvreur *et al*. 2008, Fjeldså & Bowie 2008, Blackburn & Measey 2009, Voelker *et al*. 2010, Lawson 2010, Holstein & Renner 2011, Tolley *et al*. 2011, Johnston & Anthony 2012). In particular, lineage diversification across several taxa in this region coincides with periods of uplift and aridification that predate the Pliocene (Couvreur *et al*. 2008, Fjeldså & Bowie 2008, Tolley *et al*. 2011). Likewise, studies in the Guineo-Congolian forest of West and Central Africa indicate that species richness and endemism result from the formation of the Cameroonian highlands, periods of aridification in the Miocene, and Pliocene-Pleistocene climatic cycles (*reviewed in* Plana 2004). Therefore, phylogeographic studies that investigate patterns of diversification in species across a spectrum of habitats (e.g. rainforest and savannah) may greatly improve our understanding of the dominant mechanisms shaping diversification in this biodiversity hotspot (Myers *et al*. 2000).

The Lower Guineo-Congolian forest extends across the Congo Basin from the Albertine Rift in East Africa to the Atlantic Ocean in West-Central Africa (Figure 4.1). Mounting evidence supports periods of climate-driven diversification in Guineo-Congolian rainforest plants and animals (Quérouil *et al*. 2003, Tosi 2008, Nicolas *et al*. 2010, Leaché & Fujita 2010, Born *et al*. 2010, Johnston & Anthony 2012). In particular, several studies cite genetic exchange across the northern Congo basin during more humid periods, followed by vicariance in arid periods, as a key mechanism shaping the distribution and diversity of plants, birds, and primates (Fjeldså & Lovett 1997, Couvreur *et al*. 2008, Tosi 2008). Furthermore, patterns of

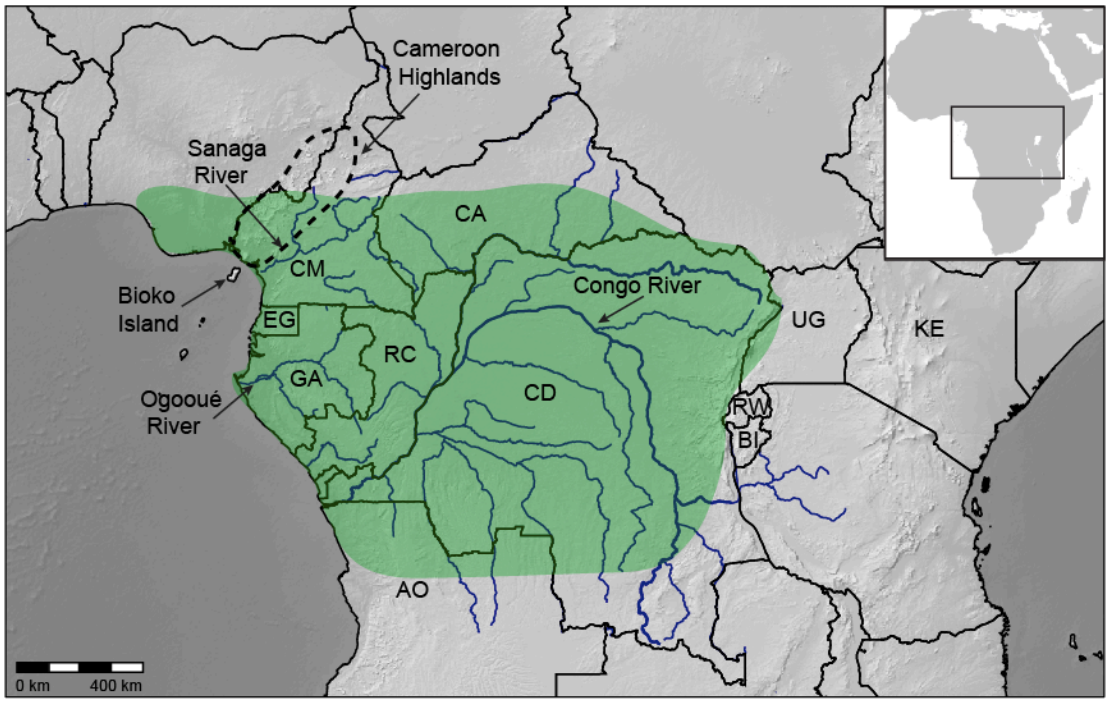

Figure 4.1 Major biogeographic features of the Lower Guineo-Congolian forest of Central Africa. Country abbreviations: AO (Angola), BI (Burundi), CA (Central African Republic), CD (Democratic Republic of Congo), CM (Cameroon), EG (Equatorial Guinea), GA (Gabon), KE (Kenya), RC (Republic of Congo), RW (Rwanda), UG (Uganda).

fine-scale phylogeographic structure in rainforest taxa are consistent with the persistence of lineages in a central refuge in the west-central Congo Basin and multiple smaller refugia throughout montane Cameroon and Gabon in western Central Africa (Tosi 2008, Nicolas *et al*. 2010, Born *et al*. 2010, Hardy *et al*. 2013). Comparisons of molecular divergence among sister species, however, reveal a continuum of divergence times ranging from Late Miocene (Holstein & Renner 2011, Duminil *et al*. 2013) to Late Pleistocene (Tosi 2008, Nicolas *et al*. 2010, Johnston & Anthony 2012) indicating that the formation of the Cameroonian Highlands and aridification during the Miocene drove earlier periods of diversification in some taxa (Duminil *et al*. 2013).

The Lower Guineo-Congolian forest also includes the land-bridge island of Bioko, which is located approximately 30 kilometres from Cameroon in West-Central Africa (Figure 4.1). Cycles of rising and retreating sea levels due to global glacial cycles in the Pliocene and Pleistocene resulted in several periods of isolation and connectivity between Bioko and Cameroon (Meyers *et al*. 1998). These recurrent cycles present multiple opportunities for genetic divergence to arise during periods of isolation followed by either homogenization or speciation when connectivity is restored. A range of divergences between populations on Bioko and their continental counterparts (Butynski & Koster 1994, Pérez *et al*. 1994, Leaché & Fujita 2010, Barej *et al*. 2014) indicates that for some taxa, populations on Bioko have remained isolated throughout cycles of isolation and connectivity with the continent, yet only 3% of the species

diversity on Bioko is endemic (Jones 1994), suggesting that gene flow during periods of connectivity often obscures population divergence (Futuyma 2010).

To investigate broad-scale patterns of diversification in the Lower Guineo-Congolian forests, we employ comparative multi-locus phylogeography in three, co-distributed species of *Hyperolius* reed frogs that inhabit a range of primary rainforest, bushland (disturbed forest) and savannah habitats. Two of these species, *H. ocellatus* and *H. tuberculatus,* also occur on Bioko Island. Phylogeography across multiple species with overlapping ranges provides a powerful approach for evaluating models of single versus multiple vicariance events across a shared landscape (Bermingham & Moritz 1998) and anuran amphibians are recognized for providing unprecedented insights into the evolutionary history of biological communities because of their low vagility and finer spatial scales of persistence (Zeisset & Beebee 2008). Specifically we aim to quantify temporal and spatial heterogeneity in divergence among our focal taxa to 1) determine whether episodes of divergence and regions of endemism in Central Africa coincide with expected vicariance events and hypothesized refugia resulting from the formation of Cameroonian highlands, Late Miocene aridification, and Pliocene-Pleistocene climatic fluctuations, and 2) quantify divergence between populations of *Hyperolius* on Bioko Island and the continent to characterize the effects of marine incursions on lineage diversification.

4.2 Material and Methods

4.2.1 Focal species and sampling details

We collected samples for our three focal taxa across their ranges in the lower Guineo-Congolian forest of Central Africa. *Hyperolius cinnamomeoventris* is a species complex that inhabits bushland and savannah habitats and includes the recently described *H. veithi* from the Congo Basin (Schick *et al*. 2010), two species endemic to the oceanic islands of São Tomé and Príncipe (*Bell et al. in review*), and highly divergent lineages in Southern Africa (*Bell et al. in review*). For this comparative study we collected 61 samples from 25 populations of the Central African distribution of the species complex (which includes *H. veithi*) and samples from the type locality of the species (Duque de Bragança, Angola; Figure 4.2a). *Hyperolius tuberculatus* occurs in bushland as well as rainforest habitats and is typically considered a single species across its range but populations in East-Central Africa have been proposed as a sister species or sub-species (*H. hutesbauti*; Schiøtz 1999). We collected 49 samples from 26 populations of *H. tuberculatus* including extensive sampling of East-Central African "*H. hutesbauti*", Bioko Island, and the type locality (Lambaréné, Gabon; Figure 4.3a). Finally, *H. ocellatus* breeds in rainforest streams and is regarded as a single but geographically variable species. We collected 70 samples from 26 populations across its range including Bioko Island (the type locality; Figure 4.4a) and populations north-west of the Sanaga River in Cameroon (Figure 4.1) that are sometimes considered a distinct sub-species (*H. o. ocellatus;* Schiøtz 1999). Tissue samples (toe clips, liver or muscle) were preserved in 95% ethanol or RNAlater and preserved specimens are accessioned in the Cornell University Museum of Vertebrates, the California Academy of Sciences, the North Carolina Museum of

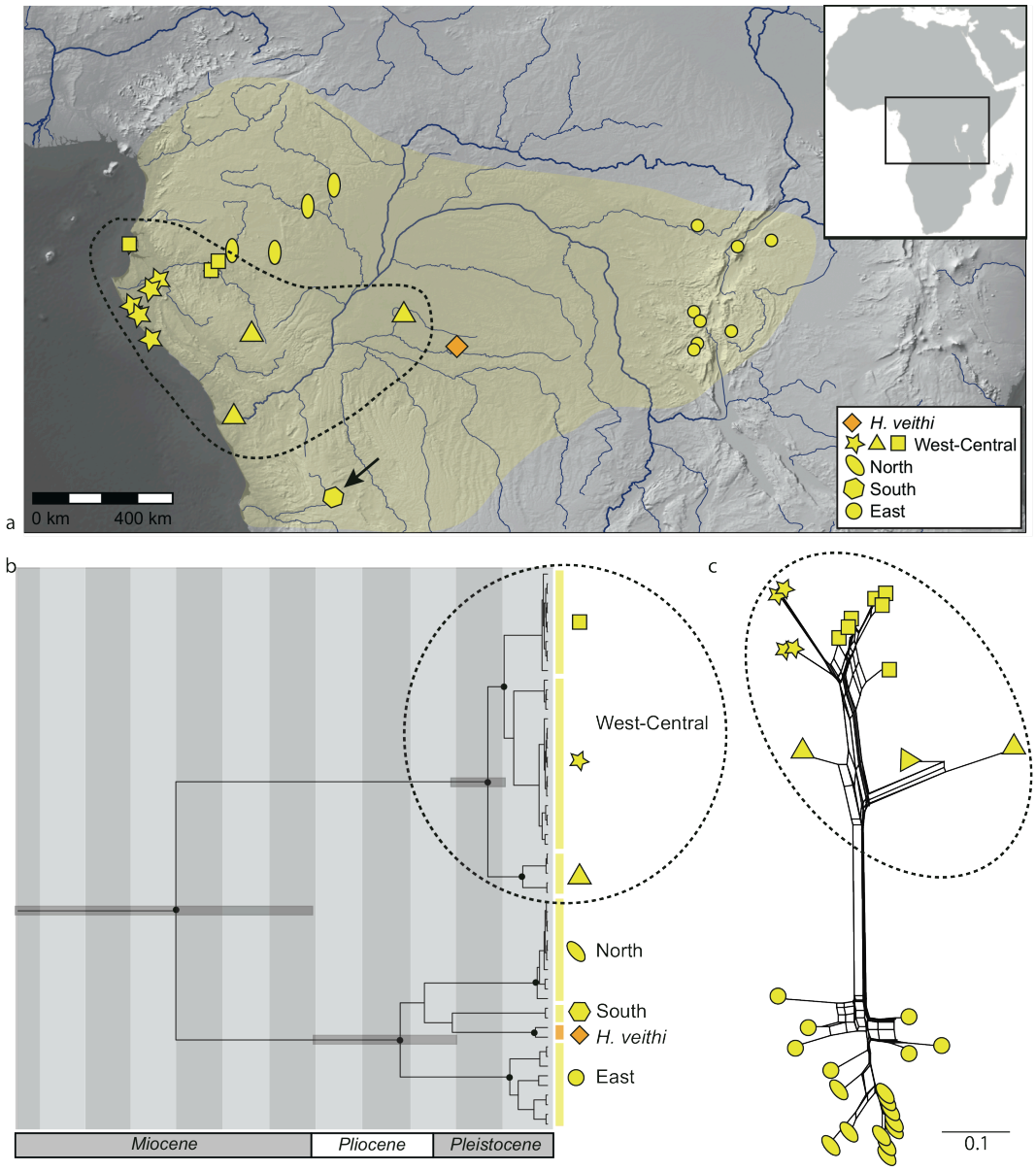

Figure 4.2 (a) Distribution of *H. cinnamomeoventris* species complex sampling localities in Central Africa. The approximate range of the *H. cinnamomeoventris* species complex is shown in yellow and the type locality for *H. cinnamomeoventris* is indicated with a black arrow. (b) Mitochondrial (*16s* and *cytochrome-b*) phylogeography. Posterior probabilities greater than 0.95 are denoted by black dots and 95% highest posterior density intervals for divergence time estimates discussed in the text are indicated. The axis indicates geological epochs (Miocene, Pliocene, and Pleistocene) and time before present in increments of one million years. (c) Multi-locus nuDNA networks generated using POFAD and SPLITSTREE. In all cases, samples are coded with shapes corresponding to mitochondrial lineages. The dashed ellipses indicate the clades referred to as "West-Central" in the text.

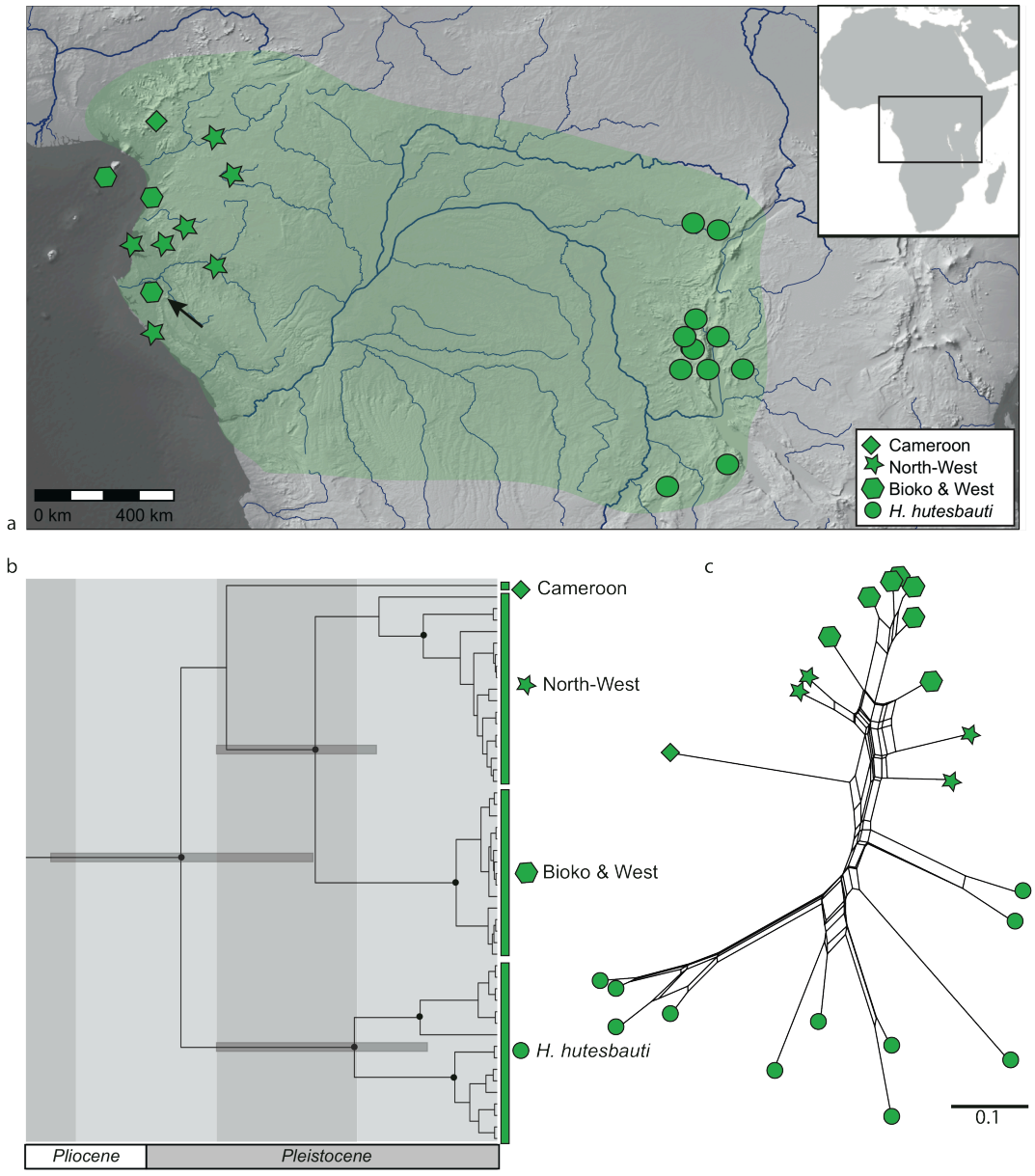

Figure 4.3 (a) Distribution of *H. tuberculatus* sampling localities in Central Africa including Bioko Island. The approximate range of *H. tuberculatus* is shown in green and the type locality is indicated with a black arrow. (b) Mitochondrial (*16s* and *cytochrome-b*) phylogeography. Posterior probabilities greater than 0.95 are denoted by black dots and 95% highest posterior density intervals for divergence time estimates discussed in the text are indicated. The axis indicates geological epochs (Pliocene, and Pleistocene) and time before present in increments of one million years. (c) Multi-locus nuDNA networks generated using POFAD and SPLITSTREE. In all cases, samples are coded with shapes corresponding to mitochondrial lineages.

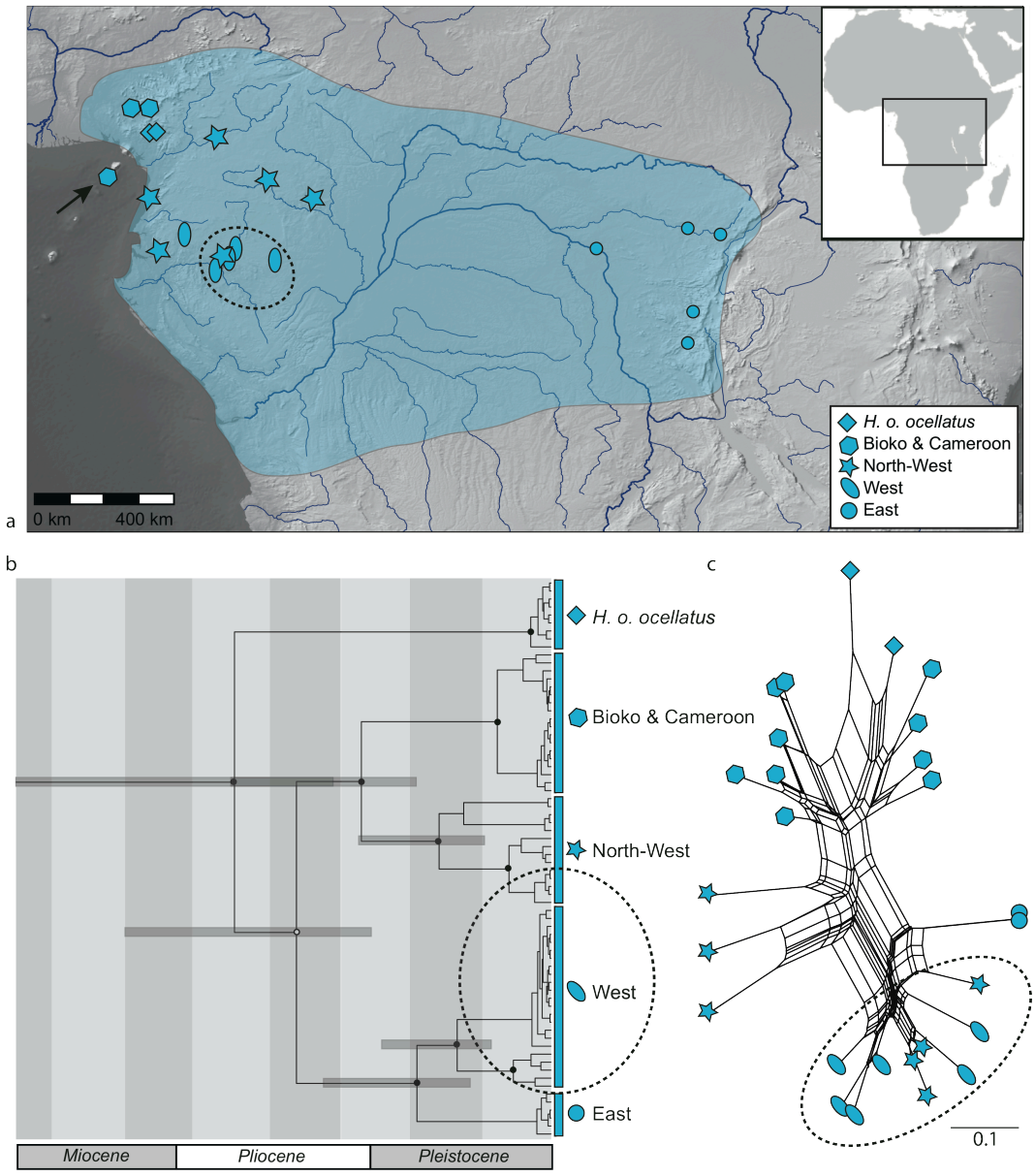

Figure 4.4 (a) Distribution of *H. ocellatus* sampling localities in Central Africa including Bioko Island. The approximate range of *H. ocellatus* is shown in blue and the type locality for *H. ocellatus* is indicated with a black arrow. (b) Mitochondrial (*16s* and *cytochrome-b*) phylogeography. Posterior probabilities greater than 0.95 are denoted by black dots, poster probabilities greater than 0.90 are denoted by open circles, and 95% highest posterior density intervals for divergence time estimates discussed in the text are indicated. The axis indicates geological epochs (Miocene, Pliocene, and Pleistocene) and time before present in increments of one million years. (c) Multi-locus nuDNA networks generated using POFAD and SPLITSTREE. In all cases, samples are coded with shapes corresponding to mitochondrial lineages. The dashed ellipses indicate the sympatric distribution of the North-West and West clades.

Natural Sciences, the Smithsonian National Museum of Natural History, the University of Texas El Paso Biodiversity Collection, the Museum of Comparative Zoology at Harvard University, the Yale Peabody Museum, the Museum of Vertebrate Zoology at the University of California Berkeley, Museum für Naturkunde in Berlin, and the National Museum in Prague, (Table 4.S1).

4.2.2. Laboratory methods

We extracted total genomic DNA using a DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA) and polymerase chain reaction (PCR)-amplified and sequenced two mitochondrial fragments (*16s* and *cytochrome-b*) and three nuclear protein-coding genes (*cmyc, pomc, Rag1*) using published primers (Table 4.S2). PCRs were carried out in a final volume of 20μL containing: 20 ng template DNA, 1× Buffer, 0.2 μM of each primer, 0.4 mM dNTP mix, and 0.125 units of *Taq* DNA polymerase (Roche Diagnostics, Indianapolis, IN, USA). Amplification was carried out with an initial denaturation for 5 min at 94 °C, followed by 35 cycles (60 s denaturation at 94 °C, 60 s annealing at 42-55°C (Table 4.S2), 60 s extension at 72 °C), and a final extension at 72 °C for 5 min. PCR products were purified using ExoSAP-IT (USB Corp., Cleveland, OH, USA), and sequenced using a BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI Automated 3730xl Genetic Analyzer (Applied Biosystems). DNA sequences were edited using SEQUENCHER 5.0.1 (Gene Codes Corp., Ann Arbor, MI, USA).

4.2.3 Mitochondrial phylogeography

Sequences were aligned using CLUSTAL X 2.0.10 (Larkin *et al*. 2007). Ambiguities in the *16s* alignment were identified by eye and excluded from subsequent analyses. We used PARTITIONFINDER 1.1.0 (Lanfear *et al*. 2012) to assign substitution models for *16s* (TrN+G, HKY+I, HKY+I) and *cytochrome b* (HKY+I, HKY, HKY+G, not partitioned by codon position), for *H. cinnamomeoventris*, *H. tuberculatus* and *H. ocellatus* respectively. For each species we inferred the mitochondrial phylogeny using Bayesian phylogenetic analyses implemented in BEAST 1.8.0 (Drummond *et al*. 2012). We chose a constant size coalescent tree prior, a strict molecular clock model, and obtained posterior distributions from two independent Markov chain Monte Carlo simulations, each run for 10 million generations, and assessed convergence with TRACER 1.5 (Rambaut *et al*. 2013). No fossils of hyperoliid frogs exist with which to calibrate divergence times, therefore we applied a constant rate of sequence divergence estimated for mitochondrial genes in tropical bufonid frogs (0.80 - 1.90% per Myr; Sanguila *et al*. 2011) to *cytochrome b*. We selected a rate prior with a mean of 1.4% and a normal distribution (95% confidence interval of 0.8 – 1.9%). The effective sample size for each parameter was well above 200 and simulations were repeated without sequence data to test the influence of priors on posterior distributions. We combined the tree files from replicate runs using LOGCOMBINER and discarded the first 10% of trees as burn-in prior to summarizing the posterior distribution of trees using TREEANNOTATOR.

4.2.4 Differentiation at nuclear loci

We collected sequences for nuclear loci from a subset of samples for each species (32 *H. cinnamomeoventris*, 23 *H. tuberculatus*, and 27 *H. ocellatus*; Table 4.S1). To check for the presence of recombination within nuclear loci we used the sum of squares method implemented in TOPALi 2 (Milne *et al*. 2008) and cropped loci to retain the largest non-recombining block. We resolved haplotypes for heterozygous individuals using PHASE v 2.1 (Stephens *et al*. 2001) implemented in DnaSP v 5.1 (Librado & Rozas 2009). To visually represent overall divergence patterns, we used a multilocus, individual-based network approach. We used PAUP v. 4.0 (Swofford 2003) to create genetic distance matrices between phased haplotypes at each locus using the HKY85 model (Hasegawa *et al*. 1985). Using POFAD v. 1.03 (Joly & Bruneau 2006) we combined individual locus matrices into one, multi-locus distance matrix (equally weighted across loci). Finally, we constructed a genetic network of the multi-locus distance matrix in SPLITSTREE v. 4.6 (Huson 2006) using the NeighborNet algorithm (Bryant 2004).

4.3 Results

4.3.1 Mitochondrial phylogeography

The mitochondrial phylogenies of the three species reveal varying levels of divergence across the Lower Guineo-Congolian forests of Central Africa. We recover deep phylogeographic structure across the *H. cinnamomeoventris* species complex with an initial divergence between the West-Central clade and rest of the Central-African range dating to the Late Miocene (Figure 4.2b) and a second divergence within the

West-Central clade dating to the Early to Mid Pleistocene (dashed ellipses Figure 4.2). The rest of the species complex includes four regional clades (a Northern clade, *H. veithi* in the Congo Basin, the type locality in Angola, and an Eastern clade) with episodes of divergence across the Congo Basin dating to the Pliocene-Pleistocene transition (Figure 4.2b). Likewise, in *H. tuberculatus* and in *H. ocellatus* we date divergence across the Congo Basin to the Pliocene-Pleistocene transition and recover three geographical clades in West-Central Africa and one clade in East-Central Africa. Populations of *H. tuberculatus* from Bioko Island are nested within a clade from coastal Cameroon and Gabon (Figure 4.3b), whereas *H. ocellatus* from Bioko Island form two distinct sub-clades that are closely related to populations in southwestern Cameroon (Figure 4.4b). Finally, populations of *H. ocellatus* north-west of the Sanaga River in Cameroon (*H. o. ocellatus*) form a distinct clade with divergence from the rest of the distribution estimated in the Late Miocene to Early Pliocene (Figure 4.4b).

4.3.2 Differentiation at nuclear loci

The multi-locus distance networks are largely congruent with the mitochondrial phylogenies in terms of locations of genetic breaks and the relative magnitude of divergence (Figures 4.2c, 4.3c, 4.4c). In *H. ocellatus*, however, the distinct mitochondrial sub-clades on Bioko Island are not differentiated at nuclear loci. Additionally, the network reveals that the North-Western and Western *H. ocellatus* mitochondrial clades are undifferentiated at nuclear loci where the clades are sympatric in Central Gabon (dashed ellipses Figure 4.4). The West-Central and Northern clades of *H. cinnamomeoventris* are sympatric in this same geographic

region, but unlike *H. ocellatus* the network indicates that the two clades are highly differentiated at nuclear loci (Figure 4.2c).

4.4 Discussion

4.4.1 Multiple episodes of climate-driven vicariance across Central Africa

Multi-locus sequence data from three co-distributed frog species demonstrates both temporal and spatial heterogeneity in diversification across the Lower Guineo-Congolian forest of Central Africa. We recover substantial phylogeographic structure in all three species and the oldest episodes of divergence date to the Late Miocene (*H. cinnamomeoventris* and *H. ocellatus*) by which time the Cameroonian Highlands and Albertine Rift had fully formed (Plana 2004). Thus diversification in our three focal species does not appear to result directly from vicariance due to the formation of the Cameroonian Highlands, however, these montane regions support altitudinal diversity, ecological gradients, and climatic refugia, all of which likely shape current patterns of diversity (Plana 2004; Hardy *et al*. 2013).

For the two species that inhabit forest and bushland habitats, *H. ocellatus* and *H. tuberculatus*, timing and degree of phylogeographic isolation correspond to species differences in dependence on rainforest habitats. In both species we recover clades in southwest Cameroon north-west of the Sanaga River, which is consistent with previous studies in rainforest trees (Dauby *et al*. 2014a) and African forest geckos (Leaché & Fujita 2010). Rivers are barriers to dispersal in many mammal species across the Guineo-Congolian forest (Quérouil *et al*. 2003, Telfer *et al*. 2003, Anthony

et al. 2007, Nicolas *et al*. 2010), but we do not recover other Central African rivers (e.g. the Congo and Ogooué Rivers; Figure 4.1) as barriers to dispersal in *Hyperolius*. Therefore this pattern likely results from the persistence of multiple small refugia along rivers and wetlands in this region (Dauby *et al*. 2014a; b). Although diversity in both species is centred in upland Cameroon and Gabon, we find evidence for multiple episodes of divergence in *H. ocellatus* throughout the Pliocene and Pleistocene whereas divergence in *H. tuberculatus* is restricted to the Pleistocene. More pronounced phylogeographic structure in *H. ocellatus*, which breeds in rainforest streams, indicates that Pliocene climatic cycles likely sundered the species range into highly divergent lineages that have not been obscured by subsequent gene flow. In contrast, *H. tuberculatus* is more tolerant of edge or open forest habitats; therefore any ancient divergences among refugial areas in this species may have become obscured by episodic introgression during rainforest expansion phases (Dynesius & Jansson 2000, Futuyma 2010). More extensive geographic sampling in this region coupled with methods that explicitly account for gene flow during periods of high connectivity may more rigorously detect differences in the species' responses to a shared climatic history (Hickerson *et al*. 2006, Leache *et al*. 2007, Dasmahapatra *et al*. 2010, Bell *et al*. 2012).

In the *H. cinnamomeoventris* species complex, which inhabits both bushland and savannah habitats, we recover deep divergence between West-Central populations and the rest of the species complex that dates to the Late Miocene, and additional periods of diversification within this clade that date to the Pleistocene. *H. cinnamomeoventris*

is one of the only *Hyperolius* to inhabit both forest and savannah habitats, and studies in other vertebrates inhabiting rainforest-savannah ecotones indicate that selection pressures in these highly unstable environments can lead to genotypic and phenotypic divergence over short evolutionary timescales (Fjeldså 1994, Smith 1997). The African Miocene was characterized by extensive aridification that resulted in the expansion of savannah habitats and dramatically restricted the distribution of rainforest habitats to small upland areas (Plana 2004). A phylogenetic study of the African forest duikers also recovers a Late Miocene origin of the sole savannah inhabiting member of the group (Johnston & Anthony 2012). Therefore, aridification in the Late Miocene may have driven initial divergence into savannah and bushland lineages of *H. cinnamomeoventris,* with subsequent interchange of savannah and rainforest habitats during the Pleistocene generating additional diversity within the West-Central clade. The presence of many divergent lineages across small spatial scales in both bushland and savannah habitats indicates that divergence in ecotones may be an important mechanism driving diversification in this group.

Despite marked differences among the three focal species in the geographic extent and temporal estimates of divergence in West-Central Africa, we recover a shared pattern of divergence between clades East and West of the Congo Basin dating to the Pliocene-Pleistocene transition. This pattern is consistent with studies in a number of plants and animals (Couvreur *et al*. 2008, Tosi 2008, Nicolas *et al*. 2010) and underscores the importance of genetic exchange across the northern Congo Basin during more humid periods, followed by divergence during arid periods, in shaping

patterns of regional diversity (Fjeldså & Lovett 1997, Tosi 2008). However, population expansion across the Congo Basin may also promote introgression between previously isolated western and eastern populations (Holstein & Renner 2011). For example, introgression between the non-sister West and North-West clades of *H. ocellatus* where they are partly sympatric in central Gabon (dashed ellipses in Figure 4.3) indicates that recent genetic divergence can become obscured when previously isolated populations come back in to contact. In contrast, the West-Central and Northern clades of *H. cinnamomeoventris* meet in the same region of central Gabon, but we do not find any evidence of introgression between these long-isolated clades. Our interpretation of these patterns is somewhat limited by a lack of sampling across much of the Congo Basin. More extensive phylogeographic sampling in this region may reveal that eastern-western phylogeographic breaks are temporally but not geographically concordant across the Congo Basin. Furthermore, deep phylogeographic structure in the *H. cinnamomeoventris* species complex across small spatial scales in this region suggests that further sampling may identify additional cryptic genetic diversity in all three species.

4.4.2 Repeated marine incursions and population divergence on the land-bridge island Bioko

As with previous phylogeographic studies of Bioko Island reptiles and amphibians (Leaché & Fujita 2010, Barej *et al*. 2014), we find moderate genetic divergence between mainland and island populations in *H. ocellatus* and *H. tuberculatus*. In both species, we recover a Late Pleistocene origin for the Bioko Island populations but

divergence in *H. ocellatus* (0.46 to 1.37 Mya) greatly predates that in *H. tuberculatus* (40 to 240 kya). The species also differ with respect to the geographic location of the most closely related mainland populations. *Hyperolius ocellatus* on Bioko are most closely related to mainland populations in southwestern Cameroon, similar to the pattern recovered in African forest geckos (*Hemidactylus fasciatus;* Leaché & Fujita 2010). In contrast, Bioko populations of *H. tuberculatus* are most closely related to mainland populations in coastal Cameroon and Gabon. Differences in the timing of divergence between island and mainland populations, as well as the geographic locations of mainland source populations, indicate that marine incursions are not the only factor restricting dispersal between Bioko and the continent. Instead, the composition of habitats connecting Bioko to the rest of the continent when sea-levels retreat may restrict dispersal across the land-bridge in some species and not in others. For instance, a patchy distribution of rainforest and bushland habitats connecting Bioko to Cameroon and Gabon may restrict dispersal in *H. ocellatus* and African forest geckos, which rely on forest habitats, but not in *H. tuberculatus*, which inhabits open forest and edge habitats. Furthermore, *H. ocellatus* and African forest geckos exhibit a fine spatial scale of population structure in montane areas in Cameroon and Gabon (Figure 4.3; Leaché & Fujita 2010), therefore sea-level incursions may simply further restrict dispersal between previously differentiated populations.

4.4.3 Lineage divergence and speciation in Central African Hyperolius

Comparative phylogeography can inform how species differ in their responses to a shared geographic and climatic history, and inform our expectations for further studies

of speciation and the evolution of reproductive isolation. For instance, lineages that diverged in response to earlier climatic or geologic processes (e.g. Pliocene or late Miocene) are expected to exhibit stronger postzygotic isolation than those that formed in response to more recent (mid-late Pleistocene) events (Avise 2000, Weir & Price 2011). The wide spectrum of divergence times across our three focal taxa, as well as regions of secondary contact between previously isolated lineages, thus present a rich comparative framework in which to investigate the accumulation of reproductive isolation and phenotypic divergence in Central African *Hyperolius*.

4.5 Acknowledgments

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Mangroves, ICCN (M. Collet) and the Royal Belgian Institute of Natural Sciences (Z.T. Nagy), and we thank Z. Chifundera Kusamba (CRSN, Lwiro) for assistance in the field. We thank K. Jackson, D. Mulcahy, C. Spencer, J. Rosado, G. Watkins-Colwell, and A. Wynn for providing additional tissue samples, and M. Hydeman for assistance in the molecular lab. Funding was provided by grants from the Explorer's Club, the American Philosophical Society, Sigma Xi, the Society of Systematic Biologists Graduate Student Research Award, National Geographic, and the Mario Einaudi Center for International Studies, the Graduate School, the Andrew W. Mellon Foundation, the Paul P. Feeny Fund, and the Paul Graduate Fellowship at Cornell University (RCB), the Museum of Comparative Zoology Herpetology Division at Harvard University (RCB & BLS), the BIOTA project (SL) of the Federal Ministry of Education and Research (BMB+F, Germany), Exxon Mobile (GH), and the Ministry of Culture of the Czech Republic (DKRVO 2014/14, National Museum, 00023272).

SUPPLEMENTAL INFORMATION FOR CHAPTER 4 - CLIMATIC REFUGIA AND MARINE INCURSIONS SHAPE DIVERSIFICATION IN CENTRAL AFRICAN REED FROGS

Table 4.S1 Sampling localities and voucher information. Abbreviations as follow: AOMA (Angola-Malanje), BICP (Burundi-Cibitoke), BIRP (Burundi-Rutana), CDBC (Dem Rep Congo-Bas-Congo), CDBP (Dem Rep Congo-Bandundu), CDKP (Dem Rep Congo-Katanga), CDNK (Dem Rep Congo-North Kivu), CDOR (Dem Rep Congo-Orientale), CDSK (Dem Rep Congo-South Kivu), CFSM (Central African Republic Sangha-Mbaéré), CMCE (Cameroon-Centre), CMEA (Cameroon-East), CMLI (Cameroon-Littoral), CMSO (Cameroon-South), CMSW (Cameroon-Southwest), CMWE (Cameroon-West), EGAB (Equatorial Guinea-Bioko-Arena Blanca), EGLL (Equatorial Guinea-Bioko-Lago Loretto), EGLU (Equatorial Guinea-Bioko-Luba), EGMO (Equatorial Guinea-Bioko-Moeri), EGMM (Equatorial Guinea-Bioko-Moka), EGPB (Equatorial Guinea-Bioko-Pico Basile), EGRI (Equatorial Guinea-Bioko-Riaba), EGWN (Equatorial Guinea-Rio Muni-Wele-Nzas), GAES (Gabon-Estuaire), GAMO (Gabon-Moyen-Ogooué), GANP (Gabon-Nyanga), GAOI (Gabon-Ogooué-Ivindo), GAOM (Gabon-Ogooué-Maritime), RCCO (Rep Congo-Cuvette-Ouest), RCLE (Rep Congo-Lekoumou), RWSO (Rwanda-Southern Province), UGWE (Uganda-Western Region)

Species	Locality	Catalog or Field No.	Lat	Long	Clade	mtDNA	nuDNA
H. cinn	AOMA	AC3008	-8.8954	16.0871	South	X	
H. cinn	AOMA	AC3017	-8.8954	16.0871	South	X	
H. cinn	CDBC	PM056	-5.8900	12.7700	West-Central	X	
H. cinn	CDBC	PM058	-5.8900	12.7700	West-Central	X	X
H. cinn	CDBP	VGCD1273	-1.9200	18.6400	West-Central		X
H. cinn	CDBP	VGCD1274	-1.9200	18.6400	West-Central		X
H. cinn	CDNK	EBG1884	0.5682	29.9178	East	X	X
H. cinn	CDNK	EBG1885	0.5682	29.9178	East	X	X
H. cinn	CDNK	EBG2305	1.4007	28.5688	East	X	X
H. cinn	CDSK	EBG1306	-1.8744	28.4524	East	X	X
H. cinn	CDSK	EBG1503	-2.2078	28.6296	East	X	X
H. cinn	CDSK	EBG2691	-3.0401	28.5050	East	X	
H. cinn	CDSK	ELI438	-3.3368	28.4223	East	X	X
H. cinn	CFSM	ds69	2.9250	16.2569	North	X	
H. cinn	CMEA	VG10194	2.1000	15.3600	North	X	X
H. cinn	GAES	NMNH578128	0.6030	9.3373	West-Central	X	
H. cinn	GAES	NMNH578129	0.6030	9.3373	West-Central	X	

Species	Locality	Catalog or Field No.	Lat	Long	Clade	mtDNA	nuDNA
H. cinn	GAES	NMNH578138	0.5736	9.3384	West-Central	X	
H. cinn	GAMO	BLS 16215	-0.6861	10.2281	West-Central	X	X
H. cinn	GAMO	BLS 16216	-0.6861	10.2281	West-Central	X	X
H. cinn	GAMO	BLS 16228	-1.1237	10.0283	West-Central	X	X
H. cinn	GAMO	BLS 16229	-1.1237	10.0283	West-Central	X	X
H. cinn	GAMO	BLS 16230	-1.1237	10.0283	West-Central	X	
H. cinn	GAMO	BLS 16306	-1.1154	10.0235	West-Central	X	
H. cinn	GAMO	BLS 16358	-0.6861	10.2281	West-Central	X	
H. cinn	GAMO	BLS 16370	-1.1086	10.0303	West-Central	X	
H. cinn	GAMO	BLS 16371	-1.1086	10.0303	West-Central	X	
H. cinn	GAMO	BLS 16372	-1.1086	10.0303	West-Central	X	
H. cinn	GAMO	BLS 16393	-1.1001	10.0276	West-Central	X	
H. cinn	GAMO	BLS 16394	-1.1001	10.0276	West-Central	X	
H. cinn	GANP	NMNH578115	-2.7868	10.0455	West-Central	X	
H. cinn	GANP	NMNH578116	-2.7868	10.0455	West-Central	X	
H. cinn	GANP	NMNH578117	-2.7868	10.0455	West-Central	X	
H. cinn	GANP	NMNH578136	-2.7868	10.0455	West-Central	X	
H. cinn	GAOI	BLS13798	0.5112	12.8028	North	X	X
H. cinn	GAOI	BLS13799	0.5112	12.8028	North	X	X
H. cinn	GAOI	BLS13800	0.5112	12.8028	North	X	X
H. cinn	GAOI	BLS13801	0.5112	12.8028	North	X	X
H. cinn	GAOI	BLS14018	0.5112	12.8028	North	X	X
H. cinn	GAOI	BLS14020	0.5112	12.8028	North	X	X
H. cinn	GAOI	BLS14129	0.5112	12.8028	North	X	X
H. cinn	GAOI	BLS14133	0.5112	12.8028	North	X	X
H. cinn	GAOI	BLS14714	-0.2095	12.2905	West-Central	X	X
H. cinn	GAOI	BLS14715	-0.2095	12.2905	West-Central	X	X
H. cinn	GAOI	BLS14717	-0.2095	12.2905	West-Central	X	X
H. cinn	GAOI	BLS14740	-0.2095	12.2889	West-Central	X	X
H. cinn	GAOI	BLS14744	-0.1956	12.1960	West-Central	X	X
H. cinn	GAOI	BLS14796	-0.0955	12.3212	West-Central	X	
H. cinn	GAOI	BLS14830	-0.1956	12.1960	West-Central	X	X
H. cinn	GAOI	BLS14831	-0.0426	12.2983	West-Central	X	X
H. cinn	GAOM	BLS14236	-1.8140	9.3556	West-Central	X	
H. cinn	GAOM	BLS14257	-1.8914	9.5682	West-Central	X	
H. cinn	RCCO	VGCG12092	0.0700	14.2400	North	X	X
H. cinn	RCCO	VGCG12093	0.0700	14.2400	North	X	X
H. cinn	RCLE	FSKJ246971	-2.7942	13.5350	West-Central	X	
H. cinn	RCLE	FSKJ246979	-2.7942	13.5350	West-Central	X	

Species	Locality	Catalog or Field No.	Lat	Long	Clade	mtDNA	nuDNA
H. cinn	RCLE	FSKJ246989	-2.7942	13.5350	West-Central	X	
H. cinn	RWSO	JMD651	-2.6011	29.7372	East	X	X
H. cinn	UGWE	SL326	0.8000	31.0667	East	X	
H. cinn	CD	A519	-2.8800	20.4100	*H. veithi*	X	
H. cinn	CD	A520	-2.8800	20.4100	*H. veithi*	X	
H. ocel	CDOR	CU15082	0.5496	25.1556	East	X	X
H. ocel	CDOR	CU15087	0.5496	25.1556	East	X	X
H. ocel	CDOR	EBG2496	1.0826	29.3900	East	X	
H. ocel	CDOR	EBG2597	1.2455	28.3434	East	X	
H. ocel	CDSK	EBG1318	-1.8744	28.4524	East	X	
H. ocel	CDSK	EBG2763	-3.0229	28.2803	East	X	
H. ocel	CMCE	CAS249970	4.6116	12.2254	North-West	X	
H. ocel	CMCE	CAS249971	4.6116	12.2254	North-West	X	
H. ocel	CMEA	VG09047	3.0900	13.8300	North-West	X	X
H. ocel	CMEA	VG10142	2.4400	15.4300	North-West	X	X
H. ocel	CMLI	MB365	4.8397	9.9303	*H. o. ocellatus*	X	
H. ocel	CMLI	MB366	4.8397	9.9303	*H. o. ocellatus*	X	
H. ocel	CMLI	MB367	4.8397	9.9303	*H. o. ocellatus*	X	
H. ocel	CMLI	MB370	4.8397	9.9303	*H. o. ocellatus*	X	
H. ocel	CMLI	MB376	4.9172	9.9892	*H. o. ocellatus*	X	
H. ocel	CMLI	MB377	4.9172	9.9892	*H. o. ocellatus*	X	
H. ocel	CMSO	MB350	2.3972	10.0452	North-West	X	
H. ocel	CMSO	MB354	2.3972	10.0452	North-West	X	
H. ocel	CMSO	MB355	2.3972	10.0452	North-West	X	
H. ocel	CMSW	MCZ136833	5.6200	9.9200	BK + CM	X	X
H. ocel	CMSW	MM030	5.7284	9.2939	BK + CM	X	
H. ocel	CMWE	MVZ234777	5.0080	10.1789	*H. o. ocellatus*	X	
H. ocel	CMWE	MVZ234779	5.0080	10.1789	*H. o. ocellatus*	X	X
H. ocel	CMWE	MVZ234782	5.0080	10.1789	*H. o. ocellatus*	X	X
H. ocel	EGAB	RCB0415	3.5258	8.5809	BK + CM	X	X
H. ocel	EGAB	RCB0416	3.5258	8.5809	BK + CM	X	X
H. ocel	EGAB	RCB0417	3.5258	8.5809	BK + CM	X	X
H. ocel	EGAB	RCB0418	3.5258	8.5809	BK + CM	X	X
H. ocel	EGLU	CAS207784	3.4830	8.5820	BK + CM	X	X
H. ocel	EGLU	CAS207785	3.4830	8.5820	BK + CM	X	X
H. ocel	EGLU	CAS207794	3.4830	8.5820	BK + CM	X	X
H. ocel	EGLU	CAS207795	3.4830	8.5820	BK + CM	X	X
H. ocel	EGMO	RCB0171	3.4673	8.6411	BK + CM	X	X
H. ocel	EGMO	RCB0214	3.4673	8.6411	BK + CM	X	

Species	Locality	Catalog or Field No.	Lat	Long	Clade	mtDNA	nuDNA
H. ocel	EGMO	RCB0217	3.4673	8.6411	BK + CM	X	
H. ocel	EGMO	RCB0218	3.4673	8.6411	BK + CM	X	
H. ocel	EGMO	RCB0221	3.4673	8.6411	BK + CM	X	
H. ocel	EGPB	CAS207829	3.7052	8.8794	BK + CM	X	
H. ocel	EGRI	RCB0396	3.3917	8.7625	BK + CM	X	
H. ocel	EGWN	A8086	*1.1708*	*11.1284*	West	X	
H. ocel	GAES	BLS13532	0.4536	10.2781	North-West	X	X
H. ocel	GAES	BLS13533	0.4536	10.2781	North-West	X	X
H. ocel	GAES	BLS13590	0.4536	10.2781	North-West	X	X
H. ocel	GAOI	BLS13756	0.5162	12.7946	West	X	X
H. ocel	GAOI	BLS13757	0.5162	12.7946	West	X	X
H. ocel	GAOI	BLS13758	0.5162	12.7946	West	X	X
H. ocel	GAOI	BLS13759	0.5162	12.7946	West	X	X
H. ocel	GAOI	BLS13826	0.4999	12.8018	West	X	X
H. ocel	GAOI	BLS13827	0.4999	12.8018	West	X	X
H. ocel	GAOI	BLS13828	0.4999	12.8018	West	X	X
H. ocel	GAOI	BLS14048	0.2938	12.5662	West	X	
H. ocel	GAOI	BLS14054	0.2938	12.5662	West	X	
H. ocel	GAOI	BLS14056	0.2938	12.5662	North-West	X	
H. ocel	GAOI	BLS14058	0.2938	12.5662	North-West	X	
H. ocel	GAOI	BLS14064	0.2938	12.5662	North-West	X	
H. ocel	GAOI	BLS14067	0.2938	12.5662	West	X	
H. ocel	GAOI	BLS14071	0.2938	12.5662	West	X	
H. ocel	GAOI	BLS14074	0.2938	12.5662	West	X	
H. ocel	GAOI	BLS14077	0.2938	12.5662	North-West	X	
H. ocel	GAOI	BLS14091	0.2927	12.5739	West	X	
H. ocel	GAOI	BLS14092	0.2927	12.5739	West	X	
H. ocel	GAOI	BLS14094	0.2927	12.5739	West	X	
H. ocel	GAOI	BLS14095	0.2927	12.5739	West	X	
H. ocel	GAOI	BLS14127	0.5112	12.8028	West	X	
H. ocel	GAOI	BLS14770	-0.0426	12.2983	West	X	
H. ocel	GAOI	BLS14786	-0.0426	12.2983	West	X	
H. ocel	GAOI	BLS14787	-0.0426	12.2983	West	X	
H. ocel	RCCO	VGCG12096	0.0600	14.2400	West	X	X
H. ocel	RCCO	VGCG12100	0.0600	14.2400	West	X	
H. tube	BICP	EBG1996	-2.8671	29.3528	*H. hutesbauti*	X	X
H. tube	BIRP	ELI994	-4.0108	30.1468	*H. hutesbauti*	X	X
H. tube	BIRP	ELI997	-4.0108	30.1468	*H. hutesbauti*	X	X
H. tube	CDKP	EBG2922	-7.7149	29.7696	*H. hutesbauti*	X	X

Species	Locality	Catalog or Field No.	Lat	Long	Clade	mtDNA	nuDNA
H. tube	CDKP	EBG2973	-7.7149	29.7696	*H. hutesbauti*	X	X
H. tube	CDKP	ELI169	-8.7190	27.4227	*H. hutesbauti*	X	X
H. tube	CDOR	EBG2318	1.1460	29.4160	*H. hutesbauti*	X	
H. tube	CDOR	EBG2526	1.4007	28.5688	*H. hutesbauti*	X	X
H. tube	CDSK	CFS1504	-3.1258	28.4150	*H. hutesbauti*	X	
H. tube	CDSK	EBG1506	-2.2078	28.6296	*H. hutesbauti*	X	X
H. tube	CDSK	EBG1657	-3.3734	28.6431	*H. hutesbauti*	X	X
H. tube	CDSK	EBG1678	-3.4039	28.5866	*H. hutesbauti*	X	
H. tube	CDSK	EBG2736	-3.0288	28.2826	*H. hutesbauti*	X	
H. tube	CDSK	ELI1305	-4.1078	29.0972	*H. hutesbauti*	X	X
H. tube	CDSK	ELI1438	-4.0901	28.1531	*H. hutesbauti*	X	X
H. tube	CDSK	ELI569	-3.3368	28.4223	*H. hutesbauti*	X	
H. tube	CMCE	CAS249988	4.6041	12.2045	North-West	X	X
H. tube	CMCE	CAS249989	4.6041	12.2045	North-West	X	X
H. tube	CMSO	MB381	2.3972	10.0452	Bioko & West	X	
H. tube	CMSO	MB384	2.3972	10.0452	Bioko & West	X	
H. tube	CMWE	MVZ234791	*5.2817*	*9.9760*	Cameroon	X	X
H. tube	EGLB	CAS207704	3.3554	8.6215	Bioko & West	X	X
H. tube	EGLB	CAS207713	3.3554	8.6215	Bioko & West	X	X
H. tube	EGLB	CAS207714	3.3554	8.6215	Bioko & West	X	X
H. tube	EGLB	RCB0016	3.3525	8.6370	Bioko & West	X	
H. tube	EGLB	RCB0017	3.3531	8.6307	Bioko & West	X	X
H. tube	EGLB	RCB0020	3.3531	8.6307	Bioko & West	X	
H. tube	EGLB	RCB0024	3.3531	8.6307	Bioko & West	X	X
H. tube	EGLL	RCB0407	3.4038	8.6691	Bioko & West	X	
H. tube	EGMM	RCB0006	3.3639	8.6580	Bioko & West	X	
H. tube	EGWN	A8062	*1.1708*	*11.1284*	North-West	X	
H. tube	GAES	BLS13669	0.6212	10.4076	North-West	X	X
H. tube	GAES	BLS13674	0.6212	10.4076	North-West	X	
H. tube	GAES	BLS13676	0.6212	10.4076	North-West	X	
H. tube	GAES	NMNH578127	0.6030	9.3373	North-West	X	
H. tube	GAES	NMNH578183	0.6030	9.3373	North-West	X	
H. tube	GAES	NMNH578184	0.6030	9.3373	North-West	X	
H. tube	GAES	NMNH578187	0.6030	9.3373	North-West	X	
H. tube	GALO	BLS 16305	-1.1078	10.0269	Bioko & West	X	X
H. tube	GALO	BLS 16327	-1.1100	10.0278	Bioko & West	X	X
H. tube	GALO	BLS 16341	-1.1403	10.0081	Bioko & West	X	
H. tube	GALO	BLS 16342	-1.1403	10.0081	Bioko & West	X	
H. tube	GALO	BLS 16387	-1.1078	10.0269	Bioko & West	X	

Species	Locality	Catalog or Field No.	Lat	Long	Clade	mtDNA	nuDNA
H. tube	GAOI	BLS14739	-0.1956	12.1960	North-West	X	X
H. tube	GAOI	BLS14789	-0.1956	12.1960	North-West	X	
H. tube	GAOI	BLS14791	-0.1956	12.1960	North-West	X	
H. tube	GAOM	NMNH578122	-2.7296	10.0188	North-West	X	
H. tube	GAOM	NMNH578123	-2.7296	10.0188	North-West	X	
H. tube	GAOM	NMNH578126	-2.7296	10.0188	North-West	X	

Table 4.S2 Primer sequences and amplification conditions for mitochondrial and nuclear sequences collected from *Hyperolius cinnamomeoventris*, *H. ocellatus*, and *H. tuberculatus*.

Primer Sequence	Length (bp)	PCR Annealing Temperature *HC*	*HO*	*HT*	Reference
16s A-L 5' CGC CTG TTT ATC AAA AAC AT 3'	521	50	50	50	(Palumbi *et al*. 1991)
16s B-H 5' CCC GTC TGA ACT CAG ATC ACG T 3'					(Palumbi *et al*. 1991)
MVZ15 5' GAA CTA ATG GCC CAC ACW WTA CG 3'	616	43*	43*	43*	(Moritz *et al*. 1992)
MVZ16 5' AAA TAG GAA RTA TCA YTC TGG TTT RAT 3'					(Moritz *et al*. 1992)
CMYC 1U 5' GAG GAC ATC TGG AAR AAR TT 3'	434	48	48	48	(Crawford 2003)
CMYC ex2dR 5' TCA TTC AAT GGG TAA GGG AAG ACC 3'					(Wiens *et al*. 2005)
POMC1 5' GAA TGT ATY AAA GMM TGC AAG ATG GWC CT 3'	521	55*	54*	54*	(Wiens *et al*. 2005)
POMC2 5' TAY TGR CCC TTY TTG TGG GCR TT 3'					(Wiens *et al*. 2005)
Rag1 F 5' GCC AGA TCT TTC ARC CAC TC 3'	467	55*	54**	55**	*L.P. Lawson*
Rag1 R 5' TGA TCT CTG GAA CRT GGG CTA 3'					*(pers comm)*

* indicates 0.3 μL of additional MgCl per reaction ** indicates 0.2 μL of additional MgCl per reaction

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