



Overseas dispersal of *Hyperolius* reed frogs from Central Africa to the oceanic islands of São Tomé and Príncipe

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ABSTRACT

Aim To infer the colonization history of reed frog species endemic to the oceanic islands of São Tomé and Príncipe, *Hyperolius mollerii* and *H. thomensis*, we quantified phylogeographical structure in the closely related *H. cinnamomeoventris* species complex, which is broadly distributed across continental Central Africa.

Location The Lower Guineo-Congolian Forest and the Gulf of Guinea islands of São Tomé and Príncipe, Central Africa.

Methods We combined gene and species tree analyses to investigate diversity and divergence among *H. cinnamomeoventris* populations, to identify the most likely dispersal route to the islands, and to infer the order in which the islands were colonized. One of the endemics (*H. mollerii*) is distributed on both islands and we quantified genetic divergence between populations.

Results We recovered three clades in *H. cinnamomeoventris* corresponding to West-Central, North/East-Central 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. mollerii* on São Tomé and Príncipe are reciprocally monophyletic at mitochondrial loci but nuclear gene trees do not support this divergence.

Main conclusions Genetic structure in the *H. cinnamomeoventris* species complex 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.

Keywords

Central Africa, Congo River, Gulf of Guinea, *Hyperolius*, long-distance dispersal, oceanic island biogeography, phylogeography, Príncipe, São Tomé, species tree.

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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 kilometres 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) Ma, 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 (Fig. 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; Loader *et al.*, 2007; Measey *et al.*, 2007; Uyeda *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, 2007; Carranza & Arnold, 2006), 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* Rapp, 1842) are thought to be the only lineage that diversified within a single island and also dispersed between islands in the archipelago: *Hyperolius thomensis* Bocage, 1886 is found only in forest habitats above 1000 m elevation on São Tomé and its putative sister taxon *Hyperolius mollerii* (Bedriaga, 1892) 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, *Hyperolius cinnamomeoventris* Bocage, 1866 (Drewes & Wilkinson, 2004; Schick *et al.*, 2010), which inhabits moist savanna, bushland, forest clearings and disturbed forest (Schjötz, 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 (Fig. 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. mollerii* populations on São Tomé and Príncipe.

MATERIALS AND METHODS

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. mollerii* from São Tomé and Príncipe (Fig. 1a). The *H. cinnamomeo-*

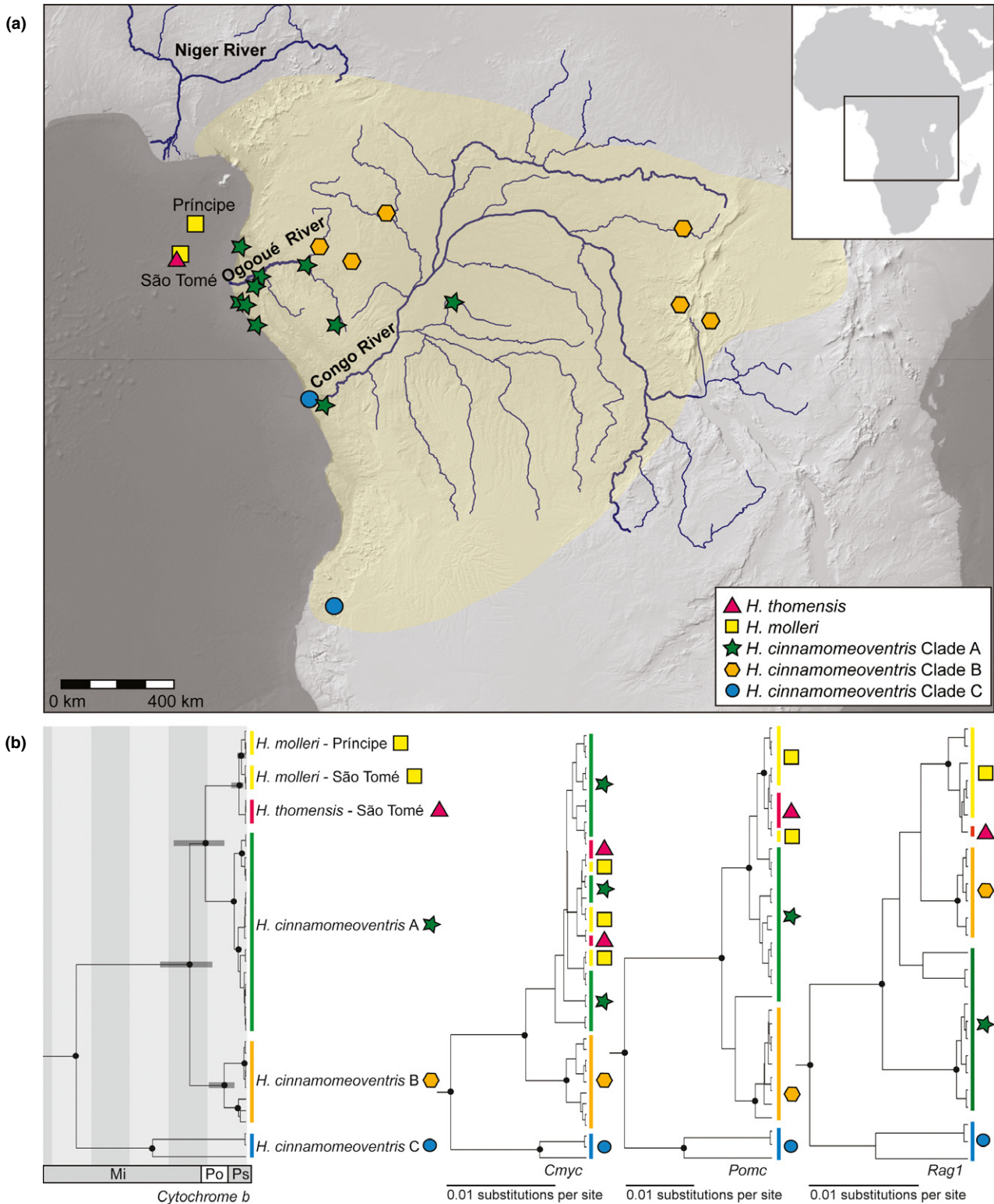


Figure 1 (a) Distribution of São Tomé and Príncipe islands (*Hyperolius thomensis* $n = 3$, *H. malleri* $n = 6$) and Central African (*H. cinnamomeoventris* species complex $n = 31$) sampling localities. Sampling localities are coloured according to mitochondrial clade and the clades supported by the *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. Clade A corresponds to West-Central Africa (the Ogooué and western Congo river basins), Clade B corresponds to North/East-Central Africa (the northern and eastern Congo River basin), and Clade C corresponds to South-Central Africa. 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.

Table 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).

Primer sequence	Locus length (bp)	PCR annealing temperature			Reference
		<i>H. cinn.</i>	<i>H. moll.</i>	<i>H. thom.</i>	
MVZ15 5' GAA CTA ATG GCC CAC ACW WTA CG 3'	616	43*	42*	42*	(Moritz <i>et al.</i> , 1992)
MVZ16 5' AAA TAG GAA RTA TCA YTC TGG TTT RAT 3'					(Moritz <i>et al.</i> , 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 <i>et al.</i> , 2005)
POMC1 5' GAA TGT ATY AAA GMM TGC AAG ATG GWC CT 3'	521	55*	52*	52*	(Wiens <i>et al.</i> , 2005)
POMC2 5' TAY TGR CCC TTY TTG TGG GCR TT 3'					(Wiens <i>et al.</i> , 2005)
RAG1 F 5' GCC AGA TCT TTC ARC CAC TC 3'	467	55*	50*	52	L.P. Lawson** (pers. comm.)
RAG1 R 5' TGA TCT CTG GAA CRT GGG CTA 3'					L.P. Lawson (pers. comm.)

*Indicates 0.3 µL of additional MgCl per reaction.

**University of Cincinnati.

ventris 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 (see Appendix S1 in Supporting Information).

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 1). PCRs were carried out in a final volume of 20 µL 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 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–KJ865980, KJ865982–KJ866052).

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 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 the number of segregating sites (θ_s) and on pairwise sequence comparisons (θ_{π}) for clades in our topology, as well as uncorrected and net sequence divergence (D_{xy} and D_a using the Tamura–Nei model; Tamura & Nei, 1993), and F_{ST} between clades.

Species tree reconstruction

Individual gene trees may differ from the underlying species tree (Maddison, 1997), so we used the multi-coalescent

Table 2 Estimates of pairwise F_{ST} values between *Hyperolius malleri* (São Tomé and Príncipe islands, $n = 6$), *H. thomensis* (São Tomé Island, $n = 3$), and the three Central African clades of the *H. cinnamomeoventris* species complex (clades A, B and C, $n = 31$) for cytochrome *b* (mtDNA) and combined nuclear loci (nDNA). Values significant at the 0.05 level are shown in bold.

	<i>H. malleri</i> Príncipe	<i>H. malleri</i> São Tomé	<i>H. thomensis</i> São Tomé	<i>H. cinnamomeoventris</i> A	<i>H. cinnamomeoventris</i> B
<i>H. malleri</i> São Tomé (mtDNA)	0.934				
<i>H. malleri</i> São Tomé (nDNA)	0.750	–			
<i>H. thomensis</i> São Tomé (mtDNA)	0.979	0.980			
<i>H. thomensis</i> São Tomé (nDNA)	0.750	0.000	–		
<i>H. cinnamomeoventris</i> A (mtDNA)	0.726	0.717	0.728		
<i>H. cinnamomeoventris</i> A (nDNA)	0.640	0.595	0.411	–	
<i>H. cinnamomeoventris</i> B (mtDNA)	0.619	0.615	0.632	0.609	
<i>H. cinnamomeoventris</i> B (nDNA)	0.900	0.900	0.880	0.797	–
<i>H. cinnamomeoventris</i> C (mtDNA)	0.737	0.727	0.729	0.778	0.645
<i>H. cinnamomeoventris</i> C (nDNA)	0.811	0.814	0.789	0.855	0.885

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 (Fig. 1b, Appendix S1). We resolved haplotypes for heterozygous individuals using PHASE 2.1 (Stephens *et al.*, 2001) implemented in DNASP 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.

RESULTS

Mitochondrial and nuclear gene trees

The mitochondrial gene tree reveals three distinct clades of *Hyperolius cinnamomeoventris* corresponding to a West-Central African clade (Clade A), a North/East-Central African clade (Clade B), and a South-Central African clade (Clade C; Fig. 1b). The two island species, *H. thomensis* and *H. malleri*, form three distinct clades (São Tomé and Príncipe *H. malleri* 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) and genetic diversity is greater in mainland than in island clades (Table 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 (Fig. 1b). Additionally, the nuclear gene trees indicate substantial incomplete lineage sorting among Clade A, Clade B and the island endemics (Fig. 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) and significant divergence from the two remaining clades ($Da = 1.0–2.8\%$, $F_{ST} = 0.789–0.900$; Table 2).

Species tree reconstruction

We assigned samples to operational species units following the geographical clades recovered in the mitochondrial gene tree (Fig. 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. malleri*) 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 (Fig. 2).

DISCUSSION

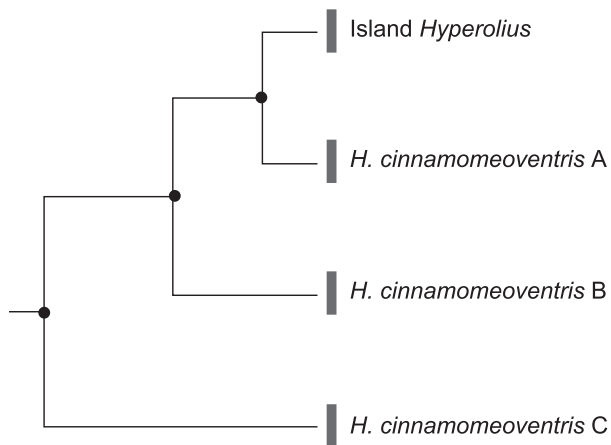
Cryptic genetic diversity in *H. cinnamomeoventris*

The *Hyperolius cinnamomeoventris* species complex is distributed in disturbed forest, moist savanna and bushland

Table 3 Summary statistics for mitochondrial and nuclear loci collected from *Hyperolius molleri* (São Tomé and Príncipe islands, $n = 6$), *H. thomensis* (São Tomé Island, $n = 3$), and the three clades of the *H. cinnamomeoventris* species complex (Central Africa, $n = 31$).

	mtDNA					nDNA			
	Samples	bp	N_h	θ_s	θ_π	Samples	bp	θ_s	θ_π
<i>H. cinnamomeoventris</i> Clade A	17	616	12	0.0486	0.0373	19	1419	0.0050	0.0022
<i>H. cinnamomeoventris</i> Clade B	8	616	6	0.0695	0.0675	9	1419	0.0023	0.0016
<i>H. cinnamomeoventris</i> Clade C	3	616	2	0.1158	0.1158	3	1419	0.0132	0.0127
<i>H. molleri</i> Príncipe	3	616	2	0.0011	0.0011	3	1419	0.0005	0.0005
<i>H. molleri</i> São Tomé	3	616	2	0.0011	0.0011	3	1419	0.0000	0.0000
<i>H. thomensis</i> São Tomé	3	616	1	0.0000	0.0000	3	1419	0.0000	0.0000

bp, sequence length in base pairs; N_h , number of haplotypes; θ_s , genetic diversity based on the number of segregating sites; θ_π , genetic diversity based on pairwise sequence comparisons.

**Figure 2** *BEAST species tree inference for nuclear (*Cmyc*, *Pomc*, *Rag1*) haplotypes collected from *Hyperolius thomensis* ($n = 3$), *H. molleri* ($n = 6$) and the *H. cinnamomeoventris* species complex ($n = 25$) from Central Africa and the islands of São Tomé and Príncipe. Posterior probabilities greater than 0.95 are denoted by black dots.

habitats across Central Africa and is hypothesized to consist of several cryptic species (Lötters *et al.*, 2004; Schick *et al.*, 2010). We recovered 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 identified 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; Fig. 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 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; Born *et al.*, 2010; Leaché & Fujita, 2010; Nicolas *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; Born *et al.*, 2010; Nicolas *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 (Born *et al.*, 2010; Nicolas *et al.*, 2010). Likewise, relatively deep divergence between eastern and western populations within Clade B (estimated at the Pliocene–Pleistocene transition; Fig. 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.

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 probably 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* probably 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 (c. 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, 1886), endemic to the island of São Tomé found strong support for a sister relationship with the *P. mascareniensis* species complex (Duméril and Bibron, 1841), 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, 1862 puddle frogs [*Phrynobatrachus dispar* (Peters, 1870), endemic to Príncipe and *Phrynobatrachus leveleve* Uyeda, Drewes and Zimkus, 2007, 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.

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. mollerii* 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; Fig. 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. mollerii* breeds near still or slow-moving water) and in morphology (*H. thomensis* is nearly twice the size of *H. mollerii*); multiple lines of evidence thus support recognizing these endemics as distinct species. Divergence between the São Tomé and Príncipe populations of *H. mollerii* is estimated to be more recent at c. 1.1 Ma to 270 ka. 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. mollerii*, and that *H. mollerii* subsequently colonized Príncipe.

Our results corroborate that the distribution of *H. mollerii* 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. mollerii* 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) is likely to pre-date the estimated age of São Tomé (13 Ma; Lee *et al.*, 1994) and the species are not each other's 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 probably not ongoing. Future studies of historical population demography of *H. mollerii* 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. mollerii* 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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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Sampling localities and voucher information.

BIOSKETCH

Rayna C. Bell is a PhD candidate at Cornell University. She is interested in mechanisms underlying diversification and speciation, especially in insular populations of tropical amphibians and reptiles. This collaboration stems from shared interests among co-authors in the natural history, population differentiation and systematics of tropical frogs.

Author contributions: R.C.B. designed the project; R.C.B., R.C.D., A.C, V.G. and B.L.S. collected field samples; R.C.B. collected and analysed the data; V.G. contributed to data analyses; all authors contributed funding to the project; and R.C.B. led the writing with help from all authors.

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