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Diversity and biogeography of frogs in the genus *Amnirana* (Anura: Ranidae) across sub-Saharan Africa

Gregory F.M. Jongsma[a,n,*], Michael F. Barej[b], Christopher D. Barratt[c], Marius Burger[d,e], Werner Conradie[f,g], Raffael Ernst[h], Eli Greenbaum[i], Mareike Hirschfeld[b], Adam D. Leaché[j], Johannes Penner[b,k], Daniel M. Portik[l], Ange-Ghislain Zassi-Boulou[m], Mark-Oliver Rödel[b], David C. Blackburn[a,n]

[a] California Academy of Sciences, San Francisco, CA 94118, USA
[b] Museum für Naturkunde, Leibniz Institute for Evolution and Biodiversity Science, Invalidenstrasse 43, 10115 Berlin, Germany
[c] University of Basel, Biogeography Research Group, Department of Environmental Sciences, Basel 4056, Switzerland
[d] African Amphibian Conservation Research Group, Unit for Environmental Sciences and Management, North-West University, Potchefstroom 2520, South Africa
[e] Flora Fauna & Man, Ecological Services Ltd., Tortola, British Virgin Island, United Kingdom
[f] Port Elizabeth Museum (Bayworld), Port Elizabeth 6013, South Africa
[g] School of Natural Resource Management, George Campus, Nelson Mandela Metropolitan University, George 6530, South Africa
[h] Senckenberg Natural History Collections Dresden, Königsbrücker Landstr. 159, D-01109 Dresden, Germany
[i] Department of Biological Sciences, University of Texas at El Paso, El Paso, TX, USA
[j] Department of Biology and Burke Museum of Natural History and Culture, University of Washington, Seattle, WA 98195-1800, USA
[k] Chair of Wildlife Ecology & Management, University of Freiburg, Tennenbacher Str. 4, 79106 Freiburg, Germany
[l] Department of Biology, University of Texas at Arlington, 501 S. Nedderman Drive, Box 19498, Arlington, TX 76019-0498, USA
[m] USA Institut national de Recherche en Sciences Exactes et Naturelles (IRSEN), BP 2400 Brazzaville, Democratic Republic of the Congo
[n] Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA

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ABSTRACT

Frogs in the genus *Amnirana* (family Ranidae) are widely distributed across sub-Saharan Africa and present a model system for exploring the relationship between diversification and geography across the continent. Using multiple loci from the mitochondrial (*16S*) and nuclear genomes (*DISP2*, *FICD*, *KIAA2013*, *REV3L*), we generated a strongly supported species-level phylogeny that provides insights into the continental biogeography of African species of *Amnirana*, which form a monophyletic group within the genus. Species delimitation analyses suggest that there may be as many as seven additional species of *Amnirana* in Africa. The biogeographic history of *Amnirana* is marked by several dispersal and vicariance events, including dispersal from the Lower Guinean Forest into the Congo Basin. In addition, phylogeographic patterns within two widespread species, *A. albolabris* and *A. galamensis*, reveal undescribed cryptic diversity. Populations assigned to *A. albolabris* in western Africa are more closely related to *A. fonensis* and require recognition as a distinct species. Our analyses reveal that the Lower and Upper Guinean Forest regions served as important centers of interspecific and intraspecific diversifications for *Amnirana*.

1. Introduction

Conducting molecular phylogenetic and phylogeographic studies at the continental scale in Africa is hampered by its large size, inaccessibility of many regions, and political instabilities. However, increased sampling over the past decade and strong global collaborations are now facilitating large-scale phylogenetic and biogeographic studies across a diversity of African taxa, including amphibians (Blackburn, 2008; Channing et al., 2016; Evans et al., 2015; Zimkus et al., 2010, 2017), reptiles (Leaché et al., 2014; Shirley et al., 2014; Medina et al., 2016; Stanley et al., 2011), mammals (Bohoussou et al., 2015; Lorenzen et al., 2012), birds (Fuchs et al., 2011; Voelker et al., 2010), dragonflies (Dijkstra, 2006), and plants (Fayolle et al., 2014; Ley et al. 2014; Dauby et al. 2014). These studies are reshaping our understanding of the processes that drive and maintain species diversity across sub-Saharan Africa as well as delimiting important areas of endemism. In addition, the cryptic species revealed by these studies are important for designating effective conservation units (Thomassen et al., 2011), and for

* Corresponding author at: Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA.
E-mail addresses: gregor.jongsma@gmail.com, gjongsma@ufl.edu (G.F.M. Jongsma).

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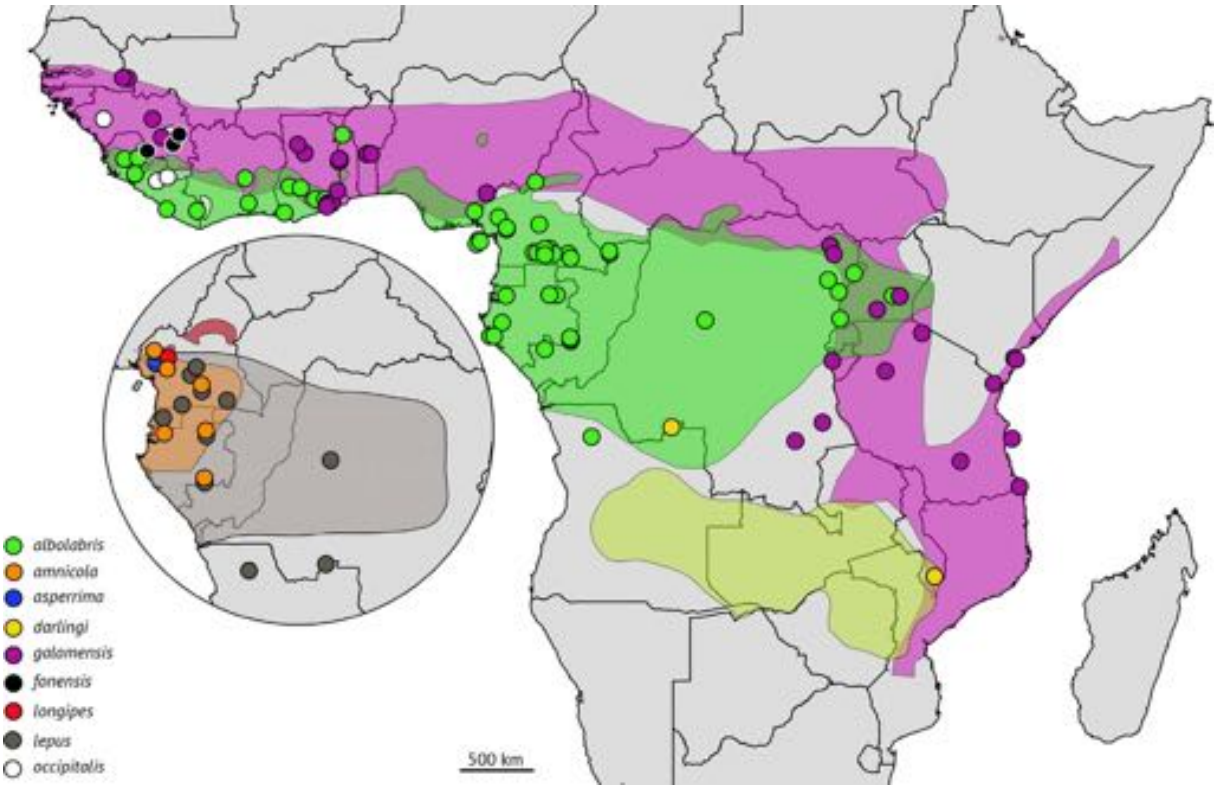

Fig. 1. Sampling localities across Africa for specimens of *Amnirana* sequenced in our study (see Appendix A) are shown for each species. For each species, the approximate distribution is depicted in the same color using IUCN maps layers. These colors match the color-coding used in Fig. 2 and Supplementary Fig. 1. Inset shows distributions of four species restricted to Central Africa and is at the same scale as the larger map. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

accurate phylogenetic estimation and hypothesis testing (Bickford et al., 2007; Heath et al., 2008; Zwickl and Hillis, 2002).

The extensive combined distribution of the eleven recognized African species in the anuran genus *Amnirana* (family Ranidae) makes this taxon ideal for investigating large-scale biogeographic patterns. Taken together, these species extend from extreme western Africa across to the Horn of Africa and south into Angola, Mozambique, Zambia, and Zimbabwe (Fig. 1). Several species have large distributions, such as *A. galamensis* and *A. albolabris*, each of which is found in more than 20 countries. These widespread species span many well-known biogeographic barriers (e.g., Congo River and Dahomey Gap) and several proposed historical forest refugia (e.g., the Cameroon Volcanic Line and the West African Nimba Mountains; Hall and Moreau, 1970; Maley, 1996). The clade comprising African *Amnirana* is embedded within a larger radiation of ranid frogs from Southeast Asia. One Asian species—*Amnirana nicobariensis*—was placed in *Amnirana* by Oliver et al. (2015), though subsequent work by Chan and Brown (2017) found that species to be closer to other Asian taxa. Our study focuses on the African members of *Amnirana* and aims to explore patterns of genetic diversity within and across major geographic regions of the continent including West, Central, and East Africa.

West Africa (west of the Nigerian Cross River) has amphibian assemblages distinct from Central Africa (Penner et al., 2011) and tends to host either divergent lineages (e.g., *Odontobatrachus*, *Pseudhymenochirus*) or relatively young species that have closely related sister species in Central Africa (Barej et al., 2014a; Blackburn, 2008; Evans et al., 2004). Two members of *Amnirana* (*A. fonensis* and *A. occidentalis*) are known only from the Upper Guinean Forests (Perret, 1983; Rödel and Bangoura, 2004). However, the divergence times of these from other species of *Amnirana* remains unclear (Kosuch et al., 2001; Oliver et al., 2015; Roelants et al., 2007; Chan and Brown, 2017). Determining whether West African taxa are early diverging lineages now restricted to this region or recent migrants from Central Africa has important implications for understanding the origins and maintenance of diversity in the Afrotropics.

East Africa hosts several centers of amphibian endemism, including the Eastern Arc Mountains (Loader et al., 2004), the Albertine Rift (Plumptre et al., 2007), and East African Coastal Forest (Burgess and Clarke, 2000). Two widespread species (*A. albolabris* and *A. galamensis*) have populations across several of these centers in East Africa (Fig. 1) and these may contain cryptic species. There is one known subspecies, *A. galamensis bravana*, known from the East African Coastal Forests of Kenya and Somalia (Poynton, 1964).

While the Lower Guinean Forest (LGF) and the Congo Basin have been largely overlooked for their potential to harbor high levels of diversity (Kingdon, 1990; Mayr and O'Hara, 1986), recent studies across several frog families demonstrate that Central Africa, and the LGF in particular, hosts high levels of diversity at both the species and population levels (Barej et al., 2010, 2011, 2014b; Blackburn, 2008, 2009, 2010; Evans et al., 2015; Hirschfeld et al., 2015; Rödel et al., 2012, 2015; Zimkus and Gvoždík, 2013; Zimkus et al., 2010). The Lower Guinean Forest harbors nearly half of the species of *Amnirana* (*A. albolabris*, *A. amnicola*, *A. asperrima*, *A. lepus*, and *A. longipes*; Perret, 1977), and therefore appears to be a center of diversity for the genus. The Plio-Pleistocene forest refuge hypothesis has been invoked to explain heightened diversity in the LGF (Anothony et al., 2007; Bouhoussen et al., 2015; Jacquet et al., 2014). Based on these studies if there is hidden diversity in *Amnirana* we predict a recent origin (i.e. since the Pliocene).

This study aims to resolve interspecific relationships of the currently recognized species of *Amnirana* found in sub-Saharan Africa. We sampled extensively across the distribution of the genus (Fig. 1), including sampling of widespread species across multiple countries, and used DNA sequence data for one mitochondrial and four nuclear genes to estimate the species-level phylogeny for African *Amnirana*. With our resolved phylogeny, we (1) evaluate cryptic species diversity, (2)

reconstruct historical biogeography and the timescale of diversification in different regions, and (3) identify important regions for future detailed study of diversification processes.

2. Materials and methods

2.1. Taxon sampling

We acquired tissues from natural history collections (Supplemental Table 1) and from our own field research. The ingroup sampling for this study includes 377 individuals representing ten of the 12 known species of *Amnirana*. Tissue samples were not available for *A. parkeriana* (Mertens, 1938), known only from the type series collected in Angola, and for *A. lemairei* (de Witte, 1921), which occurs in southern Democratic Republic of Congo, Zambia and eastern Angola. These two taxa were thus excluded from our phylogeny. Our sampling includes specimens from 22 African countries, spanning nearly the entirety of the African distribution of *Amnirana* (Fig. 1). We have samples from within 50 km of the type locality for five species (*A. albolabris*, *A. asperrima*, *A. longipes*, *A. fonensis*, *A. galamensis*) and from within 100 km for both *A. lepus* and *A. occidentalis*. Twenty-two outgroup taxa that represent nine closely related ranid genera were selected (Chen et al., 2005; Dubois, 1992), including the Asian species placed in *Amnirana*, *A. nicobariensis*, by Oliver et al. (2015). Unless otherwise stated, our taxonomy follows Frost (2017).

2.2. Extraction, amplification, and DNA sequencing

Laboratory work was conducted at the Center for Comparative Genomics (CCG) at the California Academy of Sciences (CAS). We extracted genomic DNA from tissues (liver, muscle, or toe clips) using Qiagen DNeasy Kits following their protocol for animals. Using polymerase chain reaction (PCR), we amplified a ~762 base pair (bp) fragment of mitochondrial DNA that encodes part of the mitochondrial ribosomal *16S* gene (94 °C 30 s, 52 °C 30 s, 72 °C 1 min) using 35 cycles and the oligonucleotide primers *16Sc* and *16Sd* (Moriarty and Cannatella, 2004). We also amplified four nuclear protein-coding loci (*DISP2, FICD, KIAA2013, REV3L*; Shen et al., 2013) for a subsample of 77 specimens (see Section 2.3 below) using a nested PCR protocol as outlined in Shen et al. (2013). We used ExoSAP-IT (Affymetrixs) to purify all amplified PCR products and then sequenced these in both directions by direct double-strand cycle-sequencing using BigDye v3.1 chemistry. For the mitochondrial *16S* gene, we used the amplifying primers in the cycle-sequencing reactions, while for the nuclear genes we used the universal forward and reverse sequencing primers (5′-AGGGTTTTCCCAGTCACGAC-3′ and 5′-AGATAACAATTTCACAC AGG-3′) developed by Shen et al. (2013). We then precipitated cycle sequencing products with ethanol (125 mM EDTA; pH 8.0) and sequenced these on an Applied Biosystems 3130 Genetic Analyzer. All sequences are deposited in GenBank (Supplemental Table 1).

2.3. Sequence alignment and phylogenetic analysis

For each gene region, we created contigs in Geneious v7.2 (Biomatters; http://www.geneious.com), aligned consensus sequences using MUSCLE v3.5 (Edgar, 2004), and checked manually for gross errors such as sequences in the wrong strand orientation. We retained ambiguity codes for heterozygous positions in the nuclear loci. We trimmed the resulting block of aligned sequences such that all individuals had complete data. In total there were 3196 characters included in the phylogenetic analyses, and 582 sites were variable.

We used the Akaike Information Criterion (AIC) as implemented in jModelTest v2.0 (Darriba et al., 2012) to select the best-fit model of nucleotide substitution for each gene and codon partition (Supplemental Table 2). PartitionFinder2 (Lanfear et al., 2012) was used to identify partitions. Each respective model was used in all model-based phylogenetic inferences discussed below (*16S* = GTR + I + Γ, *DISP2* = HKY + Γ, *FICD* codon 1 = K80 + Γ, *FICD* codon 2,3 = K80 + I, *KIAA2013* codon 1,2 = K80, *KIAA2013* codon 3 = K80 + Γ, *REV3L* codon 1,3 = K80, *REV3L* codon 2 = HKY + I).

We reconstructed phylogenies for each gene using maximum likelihood (ML) and Bayesian methods. Maximum likelihood analyses were conducted using RAxML v8 (Stamatakis, 2014), and Bayesian analyses were conducted using BEAST v1.8.2 (Drummond et al., 2012). The mtDNA genealogy (based on 762 bp of *16S* for 377 ingroup samples and 22 outgroup samples) was used to obtain a preliminary indication of lineage diversity. We employed the nucleotide-substitution models supported by jModelTest2 (see above), and ran an analysis for each gene in RAxML for 1000 bootstrap replicates and in BEAST for 50 million generations, sampling every 1000 generations, and assessed convergence using TRACER v1.6 (Rambaut et al., 2014).

Using the 399-sample mtDNA ML phylogeny as a guide, we selected two or more individuals for sequencing the four nuclear loci from distinct and well-supported clades (> 70 bootstrap support), as well as other samples that were either taxonomically important or represented lineages having only single samples. We sequenced this subset of 77 specimens for four nuclear loci. Our final multi-locus data matrix for mitochondrial and nuclear loci is 87.7% complete. Partitioned ML analyses were conducted on the concatenated five-locus alignment using RAxML with 2000 bootstrap replicates.

We conducted Bayesian phylogenetic analyses using BEAST for our multi-locus, mixed-model dataset, representing 77 samples. The same partitioning strategy and corresponding substitution models as in our ML analyses were used. Sampling every 1000 generations, we ran four independent Markov Chain Monte Carlo (MCMC) runs for 50 million generations. We assessed convergence of these runs using TRACER. The first 25% of sampled trees were discarded as burn-in, and we summarized the posterior distribution of trees using the maximum clade credibility tree (MCCT).

2.4. Exploratory species delimitation

To evaluate species diversity, we employed the software BPP v3.2 (Yang and Rannala, 2014; Yang, 2015). BPP is a multi-species coalescent method that uses multiple independent loci and reversible-jump Markov chain Monte Carlo (rjMCMC) to estimate the posterior probability of species delimitation hypotheses (Rannala and Yang, 2013; Yang and Rannala, 2010, 2014). We ran BPP using the joint species delimitation and species-tree inference option ('A11'; Yang, 2015). This option uses rjMCMC to collapse pre-assigned species into single species, but will not split any of the pre-assigned species into multiple species (Rannala and Yang, 2013; Yang and Rannala, 2014). We grouped individuals into minimal hypothesized species lineages based on the previously estimated phylogenetic trees (Figs. 2 and 3). We used a gamma prior for large population size (θ = G(1, 100)), and the age of the root of the species tree (τ_0 = G(2, 20), and the Dirichlet prior was set to one (Yang and Rannala, 2010). We ran analyses for 100,000 MCMC generations, sampled every 2 generations and used a 10% burn-in with the "cleandata" option. We ran two independent analyses (with random starting trees) for each scenario of 15 to 19 species, both with and without the mtDNA locus.

2.5. Molecular dating and biogeographic analyses

There are no available fossils to serve as internal calibration points within the genus *Amnirana* or within the closely related genera previously placed within *Hylarana* (Oliver et al., 2015). Therefore, we chose to estimate divergence times using previous mitochondrial substitution-rate estimates for amphibians. Because estimated mitochondrial substitution rates for *16S* range from 0.16 to 1.98% pairwise divergence per million years (Bittencourt-Silva et al., 2016; Evans et al., 2004; Fouquet et al., 2010; Lemmon et al., 2007; Macey et al., 1998,

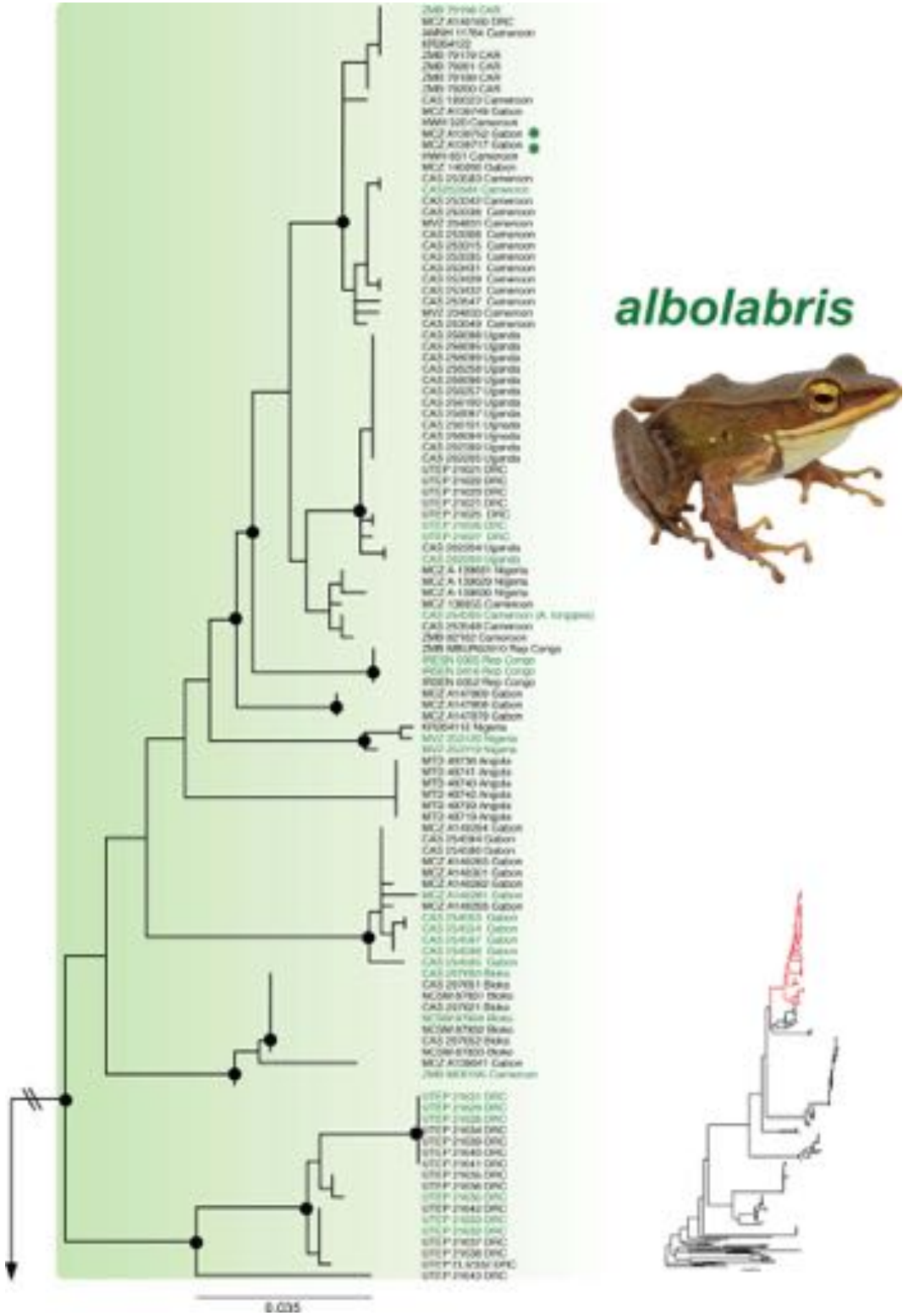

Fig. 2. ML phylogeny estimated using RAxML based on *16S* mitochondrial sequences for all samples. Black dots represent nodes with both bootstrap support above 70% and posterior probabilities above 0.95. Specimen names in the same color as clade are those selected for sequencing of nuclear loci. Asterisks indicate samples that from near or at the type locality for each species. Photographs by A. Emrich, T. Maag, and D. Portik.

2001; Monsen and Blouin, 2003; Mulcahy and Mendleson, 2000; Portillo et al., 2015; Pröhl et al., 2010), we used 1.07% (the mean of this range) to estimate divergences. Because *BEAST makes use of per-lineage rates, we divided this average pairwise divergence by two and then further divided it by 100 to express the value in substitutions/site/MYR (Heled and Drummond, 2010). We tested if our *16S* phylogeny was clock-like using Tajima's test, implemented in MEGA v7.026 (Kumar et al., 2016). This test confirmed that there are equal rates among lineages. In *BEAST, we implemented a strict clock with the per-lineage rate (0.00535 substitutions/site/MYR) and a lognormal distribution for the mtDNA region. For each of the four nuclear loci, we implemented a lognormal relaxed clock that allows branch lengths to

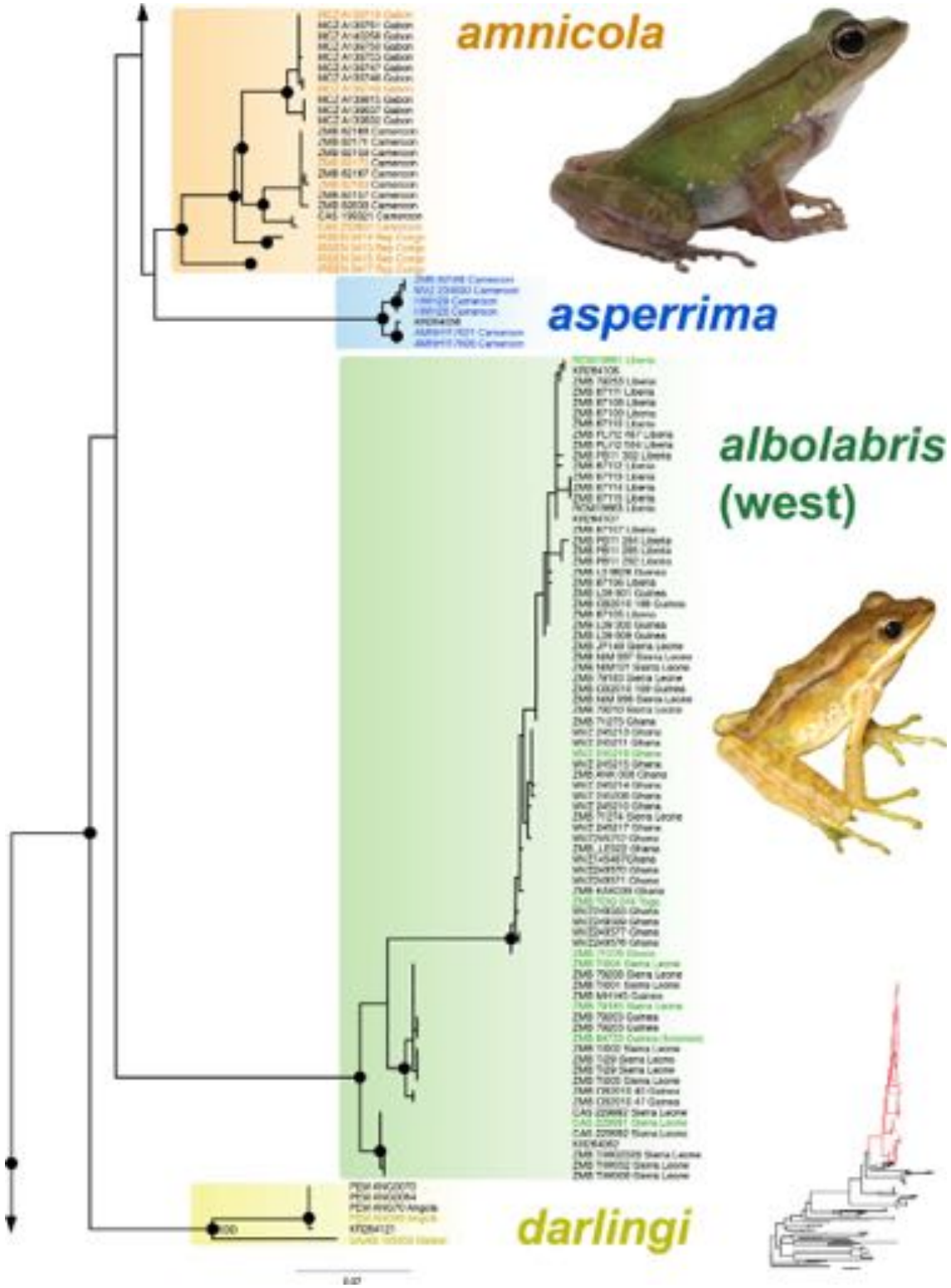

Fig. 2. (*continued*)

vary according to an uncorrelated lognormal distribution. To estimate the species-level relationships, we ran a *BEAST analysis with the seventeen species supported by the BPP analyses as the terminal taxa, and *Hylarana erythraea* and *Hydrophylax leptoglossa* as outgroups. Using our partitioned alignment for 77 specimens with mitochondrial and nuclear data, we used the same models of evolution as in the above ML and Bayesian analyses. Using a Yule prior tree, we ran four independent MCMC analyses for 100 million generations, sampling every 1000 generations, and then discarded the first 25% of samples as burn-in after confirming convergence using Tracer v1.6 (Rambaut et al., 2014). We repeated this analysis twice to confirm consistency.

Biogeographic analyses were performed using LAGRANGE (Dispersal–Extinction–Cladogenesis model; DEC; Ree and Smith, 2008) as implemented in RASP v3.1 (Yu et al., 2015). The DEC model estimates geographical range evolution using an ultrametric tree with branch lengths scaled to time and geographic areas assigned to each tip.

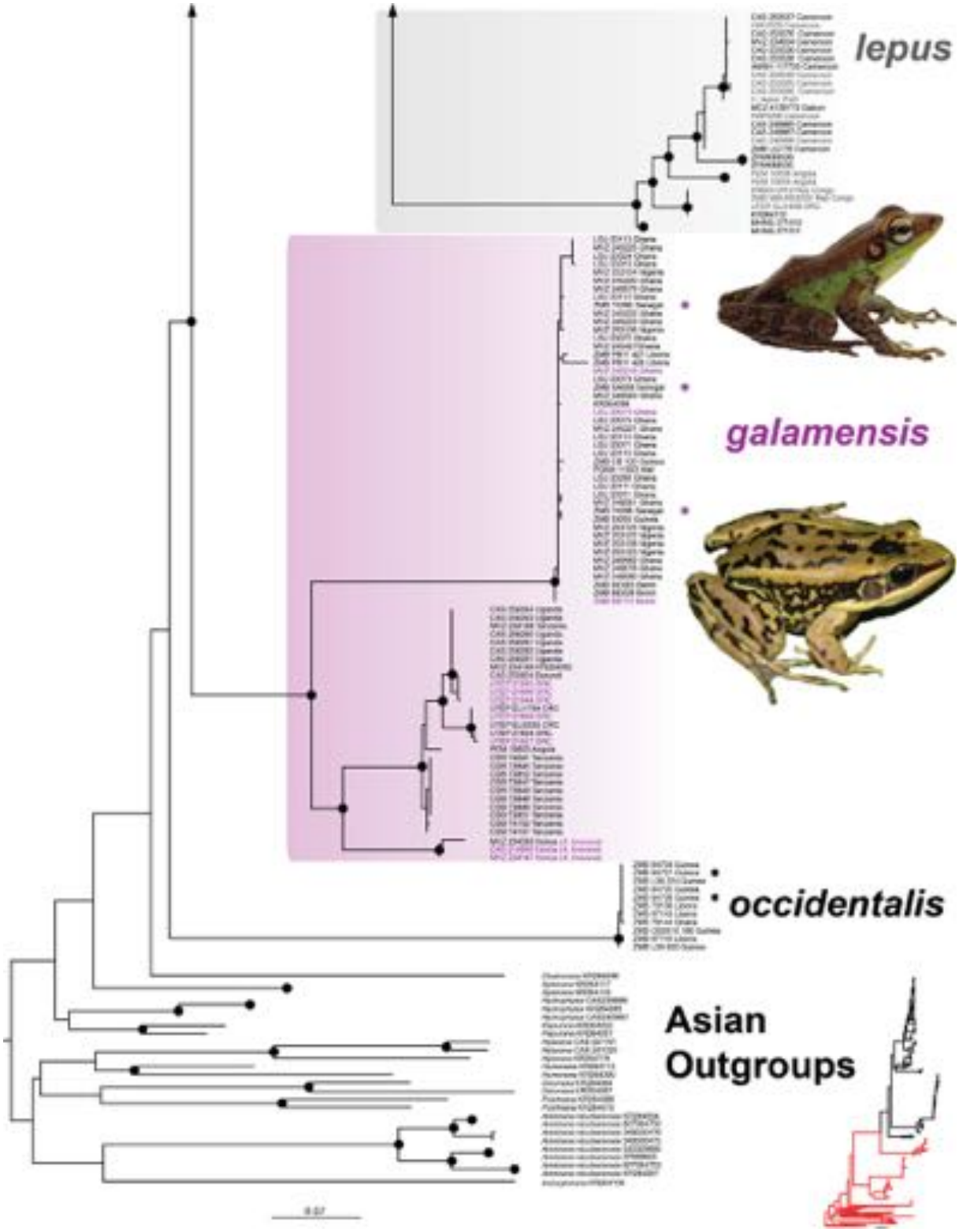

Fig. 2. (*continued*)

It computes the likelihood values of all possible ancestral distributions and uses composite Akaike weight to summarize the biogeographic reconstructions across trees. Dispersal-Extinction-Cladogenesis (DEC) utilizes a set of sampled trees, rather than a single consensus phylogeny. In RASP, we imported 100,000 trees and did not include outgroup species from Asia because our focus was on the regional biogeographic history within continental Africa, and the African species of *Amnirana* represent a single colonization of the Ranidae from Asia (Oliver et al., 2015; Fig. 2). Additionally, outgroups with large distributions may be unhelpful for biogeographic analyses and can lead to erroneous results (Buerki et al., 2011; Link-Pérez et al., 2011; Ronquist, 1997; Yu et al., 2015). Coarse geographic areas were used that correspond to biotic regions recognized by Burgess et al. (1998), including West Africa (W; west of the Nigerian Cross River), Lower Guinean Forest (G; eastern

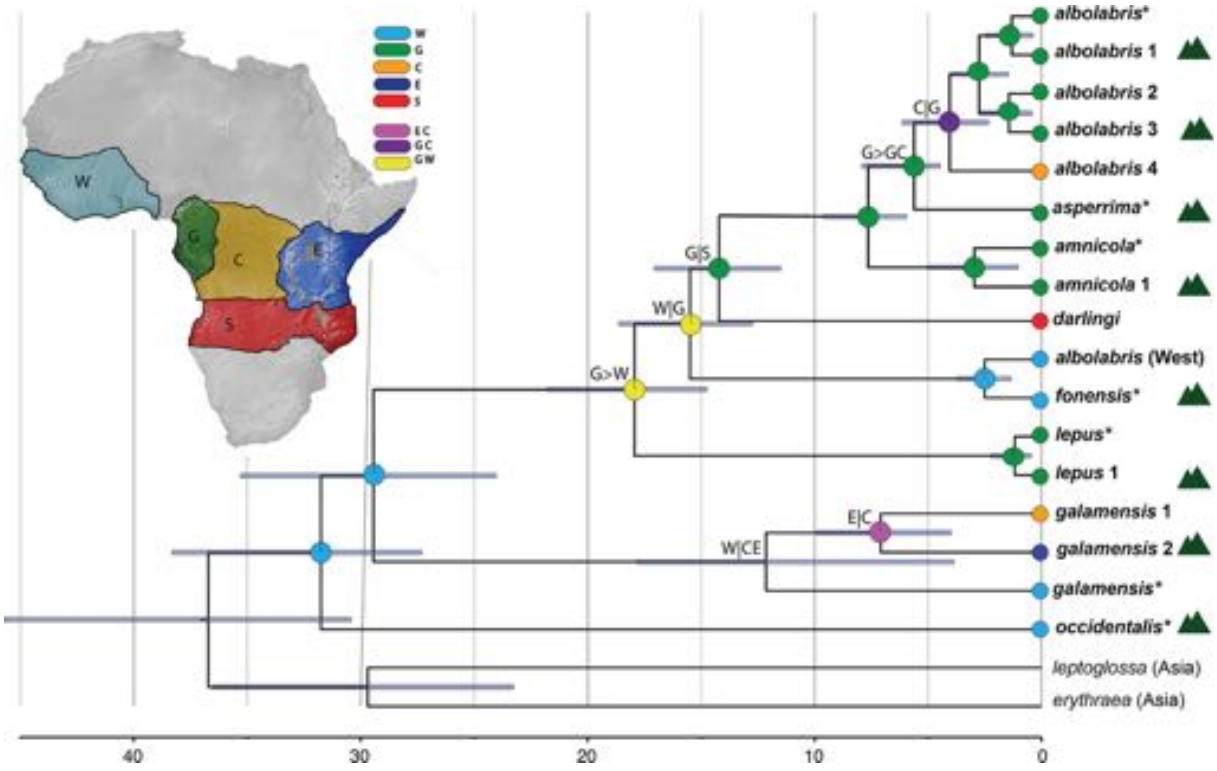

Fig. 3. Time-calibrated phylogeny from African *Amnirana* generated from *BEAST with results of Dispersal-Vicariance analysis from DEC. Major African regions: West Africa (W); Lower Guinean Forest (G); East Africa (E); and South Africa (S). The symbol "|" represents vicariance and " > " represents dispersals between regions. The node bars represent 95% credibility intervals. As in this figure, asterisks denote lineages containing specimens from at or near the type locality. Green mountains represent taxa found at known or predicted forest refugia. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Nigeria, Cameroon, Equatorial Guinea, Gabon and Southern Republic of Congo), Central Africa (C; southeastern Cameroon, southwestern Central African Republic, Republic of Congo, Democratic Republic of Congo and northern Angola), East Africa (E; Uganda, Rwanda, Burundi, Kenya, and Tanzania), and southern Africa (S; Angola, Zambia, Malawi and Mozambique) (Fig. 3). While performing ancestral-state reconstruction analyses, we did not constrain the number of unit areas allowed and used default values for other settings.

3. Results

3.1. Sequence alignment

The complete *16S* mtDNA alignment spans 762 bp for 382 ingroup samples (399 total samples). The complete alignment for the nuclear loci spans 2527 bp across four unlinked gene regions (*DISP2*, 870 bp; *FICD,* 531 bp; *KIAA2013*, 546 bp; *REV3L*, 580 bp) for 77 samples. The final concatenated alignment spans 3249 bp for 77 samples, including two outgroups.

3.2. Phylogenetic analyses

Our complete mtDNA phylogeny supports numerous clades throughout African populations of *Amnirana* (Fig. 2a–c). In the mtDNA phylogeny, we included species of nine of the ten most closely related genera recognized by Oliver et al. (2015), and the African members of *Amnirana* form a clade exclusive of these Asian lineages (Fig. 2). Based on the multi-gene, mixed-model phylogenetic analysis, *Amnirana* is monophyletic, and the African subclade is well supported (Supplementary Fig. 1). For the 77 specimens selected for sequencing nuclear loci, phylogenetic relationships were generally similar for each of these loci when analyzed separately (Supplementary Figs. 1–7).

Amnirana occidentalis, endemic to West Africa, is the sister taxon to a clade comprising all other African congeners. Within the latter clade, *A. galamensis* is the sister taxon to a clade containing all remaining African species sampled (Figs. 2 and 3). Within *A. galamensis*, the deepest phylogenetic split separates a widespread West African lineage from a clade comprising a lineage from the Kenyan coast and another that appears widespread across Eastern Africa. The remaining larger clade (i.e., *A. lepus*, *A. fonensis*, *A. albolabris*, *A. darlingi*, *A. amnicola*, and *A. asperrima*) contains mostly forest-dependent species, nearly all of which occur in the Congolian and/or the Lower Guinean Forest of Central Africa. The large-bodied *A. lepus* is the sister taxon to a clade containing *A. darlingi*, *A. fonensis*, *A. asperrima*, *A. amnicola*, *A. albolabris*, and a number of undescribed species (see below). Within West Africa (i.e., west of the Nigerian Cross River), two lineages are nested within the diversity of species from Central Africa. These lineages are currently recognized as *A. fonensis*, endemic to the Upper Guinean highlands of Guinea and Sierra Leone, and populations currently identified as *A. albolabris* that do not group with nominotypical populations from Central Africa (Perret, 1977) identified as *A. albolabris*. Based on this paraphyly, "*A. albolabris*" populations from West Africa represent an undescribed species of *Amnirana*.

3.3. Species delimitation

Our species delimitation analyses in BPP add seven species of African *Amnirana* to the existing eleven known species. *Amnirana albolabris* is revealed as diphyletic (Supplemental Table 2; Figs. 2 and 3). Samples of *A. albolabris* from the Lower Guinean Forest in Gabon (Perret, 1977) are more closely related to two species in Central Africa (*A. amnicola* and *A. asperrima*) than to the populations from West Africa that are currently termed *A. albolabris*. This West African lineage occurs in Guinea, Sierra Leone, Liberia, Côte d'Ivoire, Ghana, and Togo and is the sister lineage to *A. fonensis*. A specimen of *Amnirana longipes* (Perret, 1960) from the type locality (Bangangté, Cameroon) is not supported as

distinct by BPP analysis and is nested within a clade of nominotypical *A. albolabris* (Supplemental Table 2; Fig. 2) that, among our samples, is found in Gabon, Cameroon, Central African Republic, Democratic Republic of Congo, and Uganda (see Section 4). In addition, these analyses support several undescribed lineages within other parts of the phylogeny, including two additional species within *A. galamensis* (*galamensis* 2 and 3) in East and Central Africa, an additional species within *A. amnicola* (*amnicola* 2) and *A. lepus* (*lepus* 2), and that *A. albolabris* may represent as many as five distinct species in the LGF and Congo Basin (*albolabris* 2–5; Fig. 3; Supplemental Table 2).

3.4. Divergence times and historical biogeography

Divergences between African *Amnirana* and other members of the Ranidae most likely occurred between 30 and 40 mya (median: 33.8 mya; 95% HPD: 40.5–27.5 mya; Fig. 3). The LAGRANGE analysis provides support to eight dispersal and six vicariance events over the past 33.8 million years, including a possible origin in West Africa and subsequent dispersal across Central, East and southern Africa. The Lower Guinean Forest (LGF) region played a pivotal role in the historical diversification and biogeography of *Amnirana*. The large subclade comprising the Central African *A. albolabris* complex, *A. amnicola* complex, and *A. asperrima* originated in LGF in late Miocene (median: 8.2 mya; 95% HPD: 9.6–6.4 mya; Fig. 3). The LGF was a center of diversification with eight speciation events. West Africa (including the Upper Guinean Forest) also played an important role, hosting four speciation events. There are no known speciation events within Central, East or southern African *Amnirana*, though we note that inclusion of two species from southern Africa (*A. lemairei* and *A. parkeriana*) could change this result.

The LGF also served as an important source of diversity for other areas. For example, there were dispersal events from LGF into Central and southern Africa. These analyses support that the Dahomey Gap, which is an arid area separating the Upper and Lower Guinean forests, might have played a role in two vicariance events (1) within the *A. galamensis* complex and (2) between *A. fonensis* + *A. albolabris* (West) and the clade of *A. darlingi*, *A. amnicola*, *A. asperrima* and *A. albolabris*. Based on our dated phylogeny, there are five speciation events since the Pliocene that are associated with hypothesized forest refugia (e.g., Chaillu Massif; Born et al., 2011); this is based on counting those divergences for which the 95% confidence interval is younger than 5.2 million years (Fig. 3). Three additional speciation events are associated with forest refugia prior to the Pliocene, including the oldest African *Amnirana* lineage, *A. occidentalis*.

4. Discussion

4.1. Phylogenetic relationships and cryptic diversity

We provide the first phylogenetic study of the genus *Amnirana*. This phylogeny includes ten of the 12 recognized species, including samples that span much of the geographic distribution for even the most widespread species. Our analyses support African *Amnirana* as a monophyletic group, thus confirming Oliver et al. (2015) and Chan and Brown (2017) and suggesting a single dispersal event from Asia into Africa likely during the Late Eocene or Oligocene (Fig. 3). Our phylogenetic analyses support *A. occidentalis* from the Upper Guinean forests of West Africa as the sister taxon to all other African *Amnirana*. Both *A. occidentalis* and the *A. galamensis* species-group are highly divergent from other *Amnirana*.

Our phylogenetic and species-delimitation analyses suggest that diversity is currently underestimated for *Amnirana* across sub-Saharan Africa. BPP analysis supports seven undescribed cryptic *Amnirana* species. *Amnirana albolabris* is clearly diphyletic: one species occurs in West Africa and is the sister taxon of *A. fonensis*; the other nominotypical *A. albolabris* is widespread across the Lower Guinean Forests, Central and East Africa and it is the sister taxon of *A. asperrima* (Fig. 3). The type locality for *A. albolabris* was described as "West Africa" by Hallowell (1856), and Schmidt and Inger (1959) specified Liberia as the type locality. However, Hallowell (1856) credits Paul B. Du Chaillu as the collector, whose collections were restricted to Gabon (Du Chaillu, 1861; Perret, 1977). Furthermore, we know that Du Chaillu spent 1855–1857, north of the Ogooué River (Du Chaillu, 1861). Since the species was described in 1856, we deduce that the type locality for nominotypical *A. albolabris* is north of the Ogooué River in Gabon.

Another problematic case in the genus is *A. longipes*. Based on morphological characters distinguishing it from *A. albolabris,* Perret (1960) described *A. longipes* from high-elevation sites (> 1000 m) on the Bamiléké and Adamawa Plateaus in western Cameroon. Our phylogenetic analyses include a sample of *A. longipes* from the type locality. This specimen (CAS 254205; Bangangté, Cameroon; ♀; SVL = 68.5; foot length = 40.25) agrees with Perret's (1960) morphological description, with its proportionately longer feet relative to *A. albolabris* (♀ SVL range = 61–74 mm; ♀ foot length range: 31–36 mm). However, because this sample is nested within the Central African, nominotypical clade of *A. albolabris* ("*albolabris**" in Fig. 3), we recommend that *A. longipes* should be considered a junior synonym of *A. albolabris*.

Our species-delimitation results support that the widespread taxon *A. galamensis* comprises three distinct and geographically circumscribed species. The West African lineage is distributed from Senegal east across the Dahomey Gap to eastern Nigeria (nominotypical clade), the Central/East African lineage occurs across Burundi, Democratic Republic of Congo, Mozambique, Tanzania and Uganda ('*galamensis* 1'), and the third lineage is restricted to the Kenyan coast ('*galamensis* 2'). Despite the strong support for each of these lineages as distinct species, there is an extensive gap (~2200 km) in our sampling from a region where *A. galamensis* is known to occur. Within this sampling gap is the type locality of *Rana oubanghiensis* Mocquard, 1896, which is currently considered a junior synonym of *Amnirana galamensis*. It is possible that the Central/East African lineage ('*galamensis* 1') should be recognized as *R. oubanghiensis*. However, because we lack sampling from Central African Republic, we refrain from formally recognizing this as a distinct species. The East African coastal lineage ('*galamensis* 2') is also supported as a distinct species and was originally described as *Limnodytes bravanus* Peters, 1882 and later recognized as a subspecies of *A. galamensis* by Loveridge (1936). Based on the morphological and genealogical distinctiveness of this lineage, *Amnirana galamensis bravana* should likely be elevated to *Amnirana bravana* (Peters, 1882). However, we do not recommend doing so until further genetic data are available for regions in Central Africa, as these will inform the name that should be applied to '*galamensis* 1,' which is either an undescribed species or corresponds to *Rana oubanghiensis* Mocquard, 1896.

4.2. Divergence from Asian ancestors and early evolution in Africa

Our results suggest that the most recent common ancestor (MRCA) of extant species of African *Amnirana* occurred in the Latest Eocene or Oligocene (31.7 mya; 95% HPD: 38.1–26 mya), with the divergence from the Asian genus, *Hylarana*, 36.7 mya (median 95% HPD: 46.1–31.6 mya). These results agree with those of Bossuyt et al. (2006) and Chan and Brown (2017), but differ from those of Oliver et al. (2015) who reported an age of ~ 18.7 mya (median 95% HPD: 24–14 mya) for the MRCA of African *Amnirana*. Similarities between our divergence time estimates and those of Chan and Brown (2017) increase confidence in these estimates because of dense taxonomic sampling in their recent study.

It is surprising that the earliest phylogenetic split within African *Amnirana* separated *A. occidentalis* from an ancestor of the remaining species. This species is restricted to the Upper Guinean forests of West Africa, nearly 5000 km west of the Arabian Peninsula, through which *Amnirana* likely first colonized the African continent from Asia. This suggests that extinction events of populations in the intervening region

followed the arrival of *Amnirana* in Africa. Our DEC analysis did not detect any extinctions; however, this could be explained by the greater uncertainty in the ancestral-event estimates caused by longer branches (Ree and Sanmartín, 2009). The Upper Guinean forests are well appreciated as an important region of endemism for old lineages of amphibian taxa including *Pseudhymenochirus* (Evans et al., 2004), *Morerella* (Rödel et al., 2009), and the recently described Odontobatrachidae (Barej et al., 2014a), as well as for the mammalian tenrecoid *Micropotamogale* (Poux et al., 2008). The overall pattern of having both old and young *Amnirana* lineages in West Africa is similar to other sub-Saharan frogs including pipids (Evans et al., 2004) and arthroleptid frogs (Blackburn, 2008) and marks this region as an important refuge for ancient lineages and source of new ones.

4.3. Potential drivers of diversification events

Our results suggest that different biogeographic barriers (rivers, mountains, and arid regions) may have played an important role in shaping *Amnirana* diversity in Africa. The Dahomey Gap is an important barrier for many forest-dwelling amphibian species and appears to be a major driver of the Upper Guinean forest's biological distinctiveness from the Guineo-Congolian forest (Leaché and Fujita, 2010; Penner et al., 2011; Poynton, 1999; Schiøtz, 1967). There have been two vicariance events between West Africa and the rest of Africa.

The forest-dwelling species *Amnirana albolabris* (West) and *A. fonensis* are genetically distinct from their Central African counterparts (*A. albolabris, A. amnicola* and *A. asperrima*), whereas *A. galamensis*, a savannah specialist, shows little differentiation across the Dahomey Gap. These results support that the impact of this arid divide on the differentiation of these two lineages is attenuated by their ecological differences, suggesting that the Dahomey Gap serves as a barrier primarily for forest-dwelling species.

Rivers are also important barriers associated with lineage diversification within *Amnirana.* There are two distinct lineages within the "*albolabris*" and "*lepus*" clades, and in each case, one occurs north and the other south of the Congo River. If these were driven by the formation of the Congo River, we would expect their timing to be congruent; however, these two vicariant events did not occur simultaneously, instead occurring 4.1 mya (median 95% HPD: 4.97–3.31 mya) and 1.8 mya (median 95% HPD: 1.88–0.91 mya), respectively. There is disagreement over when the Congo River formed, and estimates vary from 34 to 0.5 mya (see Takemoto et al., 2015 for a summary). The *A. lepus* divergence across the Congo River (1.8 mya) agrees with the divergence times between chimpanzees and bonobos (Gonder et al., 2011) and between populations of the bird species *Bleda syndactyla* (Voelker et al., 2013). The incongruences in divergence times could be explained by chance events such as rafting (Bell et al., 2015) or changes in the river's course (e.g., oxbows; Toivonen et al., 2007). Similarly, the Ogooué River has driven diversification between *A. albolabris* ('*albolabris* 3' in Fig. 3) south of the Ogooué River and *A. albolabris* 2 from Cameroon Volcanic Line 2.3 mya (median 95% HPD: 3.4–1.2).

The Plio-Pleistocene Forest Refuge Hypothesis (PFRH; Haffer, 1969) has been proposed as an explanation of elevated species diversification in tropics during the past 5.3 million years (Bohoussou et al., 2015; Duminil et al., 2015; Haffer, 1969; Mittelbach et al., 2007). This hypothesis posits that as forests contract, forest-obligate populations become isolated in refugia, leading to allopatric speciation. A recent comparative phylogeographic and demographic study of two Afrotropical tree families offers high support for a scenario consistent with the PFRH (Duminil et al., 2015). The presence of distinct lineages or sympatric sister-taxa at forest refuge sites can be interpreted as the result of Plio-Pleistocene Forest Refuge action (Haffer, 1969), and several molecular studies in Africa invoke the refuge theory to explain current distributions and diversity of species (Bohoussou et al., 2015; Brouat et al., 2009; Hassanin et al., 2015; Nicolas et al., 2011; Portik et al. 2017).

Several cryptic lineages of *Amnirana* occur in predicted forest refugia. For example, *A. fonensis* and *A. albolabris* (west) occupy the Simandou and Tingi Mountains in Guinean Highlands of West Africa (Fjeldså and Lovett, 1997). In the LGF region, we detect distinct, cryptic lineages of *A. amnicola* and *A. lepus* at the Chaillu Mountains in southern Republic of Congo, and three cryptic lineages of *A. albolabris* around the Cameroon Volcanic Line (Fig. 3). The combination of our species-delimitation and divergence-time analyses support five to six speciation events occurring during the Pliocene–Pleistocene (5.3 mya–10,000 years ago). While several of these events occurred in either West or East Africa, diversification was most pronounced in the Lower Guinean Forest region, with three speciation events in the past five million years and nine speciation events total (Fig. 3).

4.4. Lower Guinean Forest diversification

Within Central Africa, the Lower Guinean Forest played a crucial role in generating and maintaining species and population-level diversity for *Amnirana*. The large "*albolabris*" subclade that includes *A. albolabris*, *A. amnicola* and *A. asperrima* originated in the LGF 7.5 mya (median 95% HPD: 9.6–5.5). Within this clade, species exhibit substantial population structure that appears largely driven by a combination of geographic barriers, especially rivers, and forest refugia, which are both found across the LGF region. While previous authors argued that Central Africa is an "evolutionary museum" dominated by old lineages (Kingdon, 1990; Mayr and O'Hara, 1986), our results support other recent studies (e.g., Anthony et al., 2007; Bohoussou et al., 2015; Evans et al., 2015; Nicolas et al., 2011; Telfer et al., 2003; Voelker et al., 2010) that demonstrate the LGF to be a rich source of diversification from the Miocene and through the Pleistocene. In addition, the LGF appears to be an important source of diversity for other regions (e.g., Blackburn, 2008; Bell et al., 2017). For example, there were two dispersal events from the LGF to the Congo Basin within both the "*albolabris*" and "*lepus*" clades (Fig. 3). Our divergence-time analyses also suggest that the widespread, nominotypical *A. albolabris* (denoted by an asterisk in Fig. 3) originated in the LGF and rapidly dispersed eastward across the Congo Basin, north of the Congo River, and into East Africa. Such a recent and rapid dispersal may explain the surprising lack of genetic structure between populations spanning over 2000 km and crossing known centers of endemism, the Cameroon Volcanic Line and Albertine Rift. The combination of stable forest refugia maintaining old lineages (Mayr and O'Hara, 1986), and both oscillating forest cover and riverine barriers driving vicariance and lineage diversification, make the LGF an important center of endemism as well as a source of species diversity in forests across western, central, and eastern Africa.

In future work, we intend to test whether phylogeographic patterns in the LGF are best explained by vicariance across rivers or isolation in and subsequent expansion from forest refugia. The LGF stands out as an ideal region to explore such questions because it contains multiple rivers that may be driving vicariant speciation (Cross, Ogooué, Sanaga Rivers; Anthony et al., 2007; Bergl and Vigilant, 2007; Missoup et al., 2009) as well as multiple postulated forest refugia (Born et al., 2011, Maley, 1996). Based on the phylogenetic framework provided here, we will expand this work to include additional amphibian taxa within the LGF and engage in spatially explicit sampling of their populations across rivers and refugia.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2017.12.006.

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