RESEARCH PAPER

Sky, sea, and forest islands: Diversification in the African leaf-folding frog Afrixalus paradorsalis (Anura: Hyperoliidae) of the Lower Guineo-Congolian rain forest

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Abstract

Aim: To investigate how putative barriers, forest refugia, and ecological gradients across the lower Guineo-Congolian rain forest shape genetic and phenotypic divergence in the leaf-folding frog *Afrixalus paradorsalis*, and examine the role of adjacent land bridge and sky-islands in diversification.

Location: The Lower Guineo-Congolian Forest, the Cameroonian Volcanic Line (CVL), and Bioko Island, Central Africa.

Taxon: Afrixalus paradorsalis (Family: Hyperoliidae), an African leaf-folding frog.

Methods: We used molecular and phenotypic data to investigate diversity and divergence among the *A. paradorsalis* species complex distributed across lowland rain forests, a land bridge island, and mountains in Central Africa. We examined the coincidence of population boundaries, landscape features, divergence times, and spatial patterns of connectivity and diversity, and subsequently performed demographic modelling using genome-wide SNP variation to distinguish among divergence mechanisms in mainland (riverine barriers, forest refugia, ecological gradients) and land bridge island populations (vicariance, overwater dispersal).

Results: We detected four genetically distinct allopatric populations corresponding to Bioko Island, the CVL, and two lowland rain forest populations split by the Sanaga River. Although lowland populations are phenotypically indistinguishable,

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pronounced body size evolution occurs at high elevation, and the timing of the formation of the high elevation population coincides with mountain uplift in the CVL. Spatial analyses and demographic modelling revealed population divergence across mainland Lower Guinea is best explained by forest refugia rather than riverine barriers or ecological gradients, and that the Bioko Island population divergence is best explained by vicariance (marine incursion) rather than overseas dispersal.

Main conclusions: We provide growing support for the important role of forest refugia in driving intraspecific divergences in the Guineo-Congolian rain forest. In *A. paradorsalis*, sky-islands in the CVL have resulted in greater genetic and phenotypic divergences than marine incursions of the land bridge Bioko Island, highlighting important differences in patterns of island-driven diversification in Lower Guinea.

KEYWORDS

Africa, amphibian, historical demography, land bridge island, Lower Guinea, phylogeography

1 | INTRODUCTION

The Guineo-Congolian rain forests of West and Central Africa are home to approximately one fifth of the world's terrestrial biodiversity (Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000), with up to 80% of this diversity considered endemic (Plana, 2004). The highest species richness in the Guineo-Congolian hotspot is centered along the Cameroonian Volcanic Line (CVL), a chain of volcanoes that comprise the Cameroonian Highland mountain ranges and the Gulf of Guinea archipelago, which includes a land bridge island (Bioko) and three oceanic islands (Príncipe, São Tomé and Annobón; Figure 1). These volcanic peaks of the CVL function as "sky islands" with steep elevational gradients that act as barriers to dispersal between surrounding lowland forests, and this region is characterized by high diversity of montane and submontane endemics (Oates, Bergl, & Linder, 2004). The land bridge island and lowland forest habitats of the Guineo-Congolian hotspot also harbour exceptional species diversity (Jones, 1994; Stuart, Adams, & Jenkins, 1990). However, the mechanisms that mediate exchanges across montane, lowland, and land bridge island habitats and how these dynamics contribute to subsequent diversification are still poorly understood.

Climatic refugia, riverine barriers, and ecological gradients all potentially contribute to diversification in tropical forests (Haffer, 1997; Moritz, Patton, Schneider, & Smith, 2000; Plana, 2004; Smith, Wayne, Girman, & Bruford, 1997). Using phylogeographical patterns to infer which of these mechanisms contributes to population divergence can be problematic when the relevant landscape features have overlapping geographical arrangements. For example, several large rivers appear to act as barriers to dispersal (the Sanaga, Congo and Ogooué: Figure 1), yet these patterns may be confounded by the historical presence of lowland rain forest climatic refugia on either side of the rivers (reviewed in Portik et al., 2017). However, population divergence due to refugia, rivers or ecological gradients should result in different population demographic histories, and these corresponding demographic predictions can be examined in a model-testing framework to improve inferences about diversification mechanisms (Portik et al., 2017). The Lower Guinea region also contains several forest-specific ecotones, which can promote parapatric speciation through disruptive selection across ecological gradients (Moritz et al., 2000; Smith et al., 1997). The longitudinal E-W gradient in precipitation across Lower Guinea (Olson et al., 2001) and the north-south climate hinge (latitude 2° N; Leroux, 1983) both coincide with population structuring in plants (reviewed in Hardy et al., 2013; Heuertz, Duminil, Dauby, Savolainen, & Hardy, 2014). Population boundaries along ecotones provide indirect evidence of the ecological gradient hypothesis, though other landscape features such as the historical presence of climatic refugia or riverine barriers can confound these patterns. Consequently, methods that explicitly quantify demographic changes in population size and gene flow may better differentiate between climatic refugia, riverine barriers, and ecological gradients as potential diversification mechanisms (Portik et al., 2017).

Cycles of isolation and connectivity on land bridge islands can also contribute to diversification in tropical forests of Lower Guinea (Bell et al., 2017; Leaché, Fujita, Minin, & Bouckaert, 2014). However, relying on phylogeographical patterns to infer the timing and duration of vicariance during marine incursions can be challenging. For example, Bioko Island is composed of three volcanic peaks on the continental shelf that range in age from 1–3 Myr (Marzoli et al., 2000) and is approximately 30 km from the present-day coast of Cameroon (Figure 1). Rising and retreating sea levels have resulted in multiple periods of isolation and connectivity, and patterns of molecular divergence between populations on Bioko and the adjacent mainland are consistent with reduced gene flow across the present-day marine barrier, particularly for amphibians and other organisms that are poor overseas dispersers (Barej et al., 2014; Bell et al., 2017; Leaché et al., 2014). Yet amphibians from multiple





FIGURE 1 Sampling localities of *Afrixalus paradorsalis paradorsalis* (circles) and *A. p. manengubensis* (triangles) in the Lower Guinean forests of continental Central Africa and Bioko Island. Sampling localities are coloured according to the mitochondrial (mtDNA) haplotype groups and distinct genetic lineages identified in analyses of the ddRADseq (nuDNA) dataset (Figure 2). Symbols with white borders reflect localities with only mtDNA sequence data and symbols with no border reflect localities with only morphological data. The right panel depicts the locations of key mountains along the Cameroon Volcanic Line

families colonized two oceanic islands in the Gulf of Guinea archipelago via overseas dispersal (Bell, Drewes, Channing, et al., 2015; Measey et al., 2007) indicating that these "sweepstakes" overseas dispersal events have occurred multiple times in the archipelago. Thus, a model-testing framework that accounts for population demographic signatures characteristic of population vicariance, founding events, and intermittent dispersal may greatly improve inferences about the evolutionary history of land bridge island populations.

Here, we investigate how putative barriers, refugia, and ecological gradients across the Guineo-Congolian rain forest shape genetic and phenotypic divergence in the leaf-folding frog Afrixalus paradorsalis Perret, 1960, a tree frog species complex comprised of two subspecies. The nominate form, A. p. paradorsalis, has a broad distribution across lowland forests throughout Lower Guinea, including Cameroon, Gabon, Equatorial Guinea and the Republic of Congo, and also occurs on Bioko Island. A morphologically distinct population in the submontane region surrounding Mt. Manengouba in the CVL (Figure 1) was previously described as the subspecies A. p. manengubensis Amiet, 2009. We use the A. paradorsalis species complex to investigate the following questions about diversification mechanisms in the Guineo-Congolian forests: (a) How many distinct populations comprise A. paradorsalis? (b) How are these populations related? (c) Do population boundaries coincide with geographic features and effective migration? (d) What demographic mechanisms have played a role in population diversity and divergence? (e) Does phenotypic variation (body size and colour pattern) reflect the taxonomic diversity detected by genetic data?

2 | MATERIALS AND METHODS

2.1 Sampling

We obtained 140 genetic samples of *Afrixalus paradorsalis* (138 A. *p. paradorsalis* and two A. *p. manengubensis*) from 29 localities across Cameroon, Gabon, Equatorial Guinea, and Republic of the Congo (Figure 1). Based on the current understanding of phylogenetic relationships within the genus *Afrixalus* (Portik, 2015), we included two samples of *A. osorioi* as an outgroup in our mtDNA analyses. Tissue samples (including liver, muscle, or toe clips) were preserved in 95% ethanol or RNA later. Specimen vouchers are deposited in natural history museum collections (Appendix S1).

2.2 | Molecular data collection

We extracted DNA, collected 16S ribosomal RNA (16S) mitochondrial sequence data, and used the double-digest RADseq laboratory protocol (ddRADseq; Peterson, Weber, Kay, Fisher, & Hoekstra, 2012) to collect genome-wide SNP data as described in Appendix S3. We processed ddRADseq data using the process_radtags module of the sTACKS 1.35 workflow (Catchen, Amores, Hohenlohe, Cresko, & Postlethwait, 2011; Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013), which demultiplexes pooled reads and performs quality filtering. We then used USTACKS to align short reads and assemble them into sets of loci, requiring a minimum depth of coverage of five reads and a maximum of two discrepancies. We generated a catalog of consensus loci using CSTACKS, and matched the loci ILEY Journal of Biogeography

from USTACKS to this catalog using SSTACKS. The POPULATIONS module was then used to generate alleles for loci present in 75% of all individuals, which resulted in a dataset of 3,968 loci. We further pruned this dataset to retain only variant and biallelic loci. If a locus contained multiple SNPs, a single site was chosen at random and retained for all subsequent analyses. Our final unlinked SNP dataset consisted of 3,917 loci for 50 individuals and is available on the Open Science Framework (OSF) (https://osf.io/fvh9k). The mtDNA sequences generated for this study are deposited in GenBank (accession numbers: MH378334-378405).

2.3 Phylogeographical structure and divergence dating

We conducted Bayesian divergence-dating analyses with our mtDNA dataset (*16S*) using BEAST 1.8 (Drummond, Suchard, Xie, & Rambaut, 2012). We used MAFFT 5 (Katoh, Kuma, Toh, & Miyata, 2005) with the E-INS-I algorithm to align *16S* sequences. We performed analyses using a GTR substitution model, and a constant size coalescent tree prior with a strict molecular clock calibrated with an amphibian rate of 2% per Myr rate of divergence (Crawford, 2003). Analyses were run for 100 million generations with sampling every 5,000 generations, producing a total of 20,000 trees. We examined convergence using TRACER 1.6 (Rambaut, Drummond, & Suchard, 2013), discarded a burn-in of 25% and generated a maximum clade credibility tree from 15,000 trees.

For our SNP dataset, we performed a discriminant analysis of principle components to identify genetic clusters of individuals (DAPC; Jombart, Devillard, & Balloux, 2010) using the R package "adegenet" 1.8 (Jombart, 2008; Jombart & Ahmed, 2011) implemented in RStudio (0.99.903). We determined the optimal number of clusters by running a principle components analysis and calculating the Bayesian information criterion (BIC; Schwarz, 1978) for sequential k-values after the retention of 100 principle components. To minimize over-fitting, an initial DAPC was used to find the a-score for each set of clusters and this value was used to select the number of principal components to retain in a subsequent re-analysis (Jombart, 2008; Jombart & Ahmed, 2011). We also performed hierarchical population-clustering analyses using the maximum likelihood approach of ADMIXTURE (Alexander, Novembre, & Lange, 2009). We used five replicates to evaluate up to six discrete populations per analysis, and subsequently determined the K value with the lowest cross-validation error. Preliminary results indicated the two samples of A. p. manengubensis were highly distinct and they were removed from hierarchical clustering for A. p. paradorsalis.

We investigated the evolutionary relationships among populations using the SNP data in a coalescent framework with SNAPP 1.3 (Bryant, Bouckaert, Felsenstein, Rosenberg, & RoyChoudhury, 2012) implemented in BEAST2 2.4 (Bouckaert et al., 2014). The SNAPP model is based on the coalescent process and therefore assumes that shared polymorphisms among lineages are due to incomplete lineage sorting and not gene flow (Bryant et al., 2012). We subsampled each distinct population (determined by DAPC and clustering analyses) to include 2–6 representatives, for a total of 19 samples, estimated mutation rates (*u* and *v*) from the data (0.997 and 1.002, respectively), and fixed the birth rate (λ) of the Yule prior to 25. We performed two independent runs with a chain length of one million generations, sampling every thousand generations. We examined convergence using TRACER 1.6 (Rambaut et al., 2013) and created a maximum clade credibility tree from the post-burn-in sample. To obtain a rough estimate of divergence dates we converted branch lengths using a human mutation rate of 1 × 10⁻⁸ (Lynch, 2010), as no estimate currently exists for amphibians.

2.4 Molecular diversity and spatial connectivity

To assess genetic diversity and population divergence in the mtDNA and SNP data, we used ARLEQUIN 3.5.2 (Excoffier & Schneider, 2005) to calculate nucleotide diversity—number of segregating sites (θ_s) and pairwise sequence comparisons (θ_π), and expected homozygosity (θ_H) for the distinct lineages. For the ddRADseq dataset we included loci that were present in at least 75% of individuals in a lineage. We also computed pairwise F_{ST} for both the mtDNA and SNP data using group assignments supported by the 16S gene tree, population clustering methods, and the DAPC.

We visualized spatial patterns of gene flow and genetic diversity using EEMS (ESTIMATED EFFECTIVE MIGRATION SURFACES; Petkova, Novembre, & Stephens, 2016), a method that uses genome-wide SNP variation and locality information to highlight regions where genetic similarity decays more quickly than expected under isolation by distance. The number of migration routes and deme sizes is specified through a grid size, and resistance distance is used to approximate the expected dissimilarity between two samples. These estimates are interpolated across geographical space to provide a visualization of levels of gene flow and diversity across regions. We chose a deme size of 700 and ran three independent analyses using RUNEEMS_SNPS, with a burn-in of 1,000,000 and MCMC length of 20,000,000. We combined the three independent runs per deme size, assessed convergence, and generated surfaces of effective diversity (q) and effective migration rates (m) using the "*R EEMS Plots*" R package (Petkova et al., 2016).

2.5 | Demographic modelling

To investigate alternative divergence scenarios for lineages of A. *p. paradorsalis*, we used the diffusion approximation method of $\delta a \delta i$ to analyse two-dimensional joint site frequency spectra (2D-JSFS; Gutenkunst, Hernandez, Williamson, & Bustamante, 2009). We examined several models representing forest refugia, riverine barrier or ecological gradient divergence scenarios using an established 2D analysis pipeline (Portik et al., 2017), and created a new set of models to explicitly investigate divergence events for mainland and island lineages. Before creating our site frequency spectra, we further filtered our ddRADseq data using a minimum minor allele frequency threshold (0.05) in the POPULATIONS module of STACKS (Catchen et al., 2011, 2013). For all analyses we projected down samples to reduce missing data and maximize the number of segregating sites for

analyses, resulting in the following allele numbers: northern, 16 alleles; southern, 32 alleles; Bioko Island, 18 alleles. Our small sample size for *A. p. manengubensis* prevented us from including this lineage in the demographic modelling.

To determine whether the joint demographic history between the northern and southern populations was best captured by divergence resulting from forest refugia, riverine barriers or ecological gradients, we carried out 2D analyses using a set of 21 demographic models (Appendix S2). In addition to the 15 models described by Portik et al. (2017), we defined four, three-epoch forest refugia models that consist of divergence in isolation, followed by secondary contact with or without instantaneous size change, followed by isolation. We also included two, two-epoch models of continuous gene flow that allow different migration rates within each epoch. To determine if pure vicariance or a founder event better explained the demographic history of the Bioko Island lineage, we generated additional types of island demographic models (Appendix S2). The vicariance models did not include changes in population size, whereas founder event models enforced exponential growth in the island population. For models in both the vicariance and founder event categories, we included a variable s that defines the fraction of the ancestral population (nuA) founding each daughter population, where nuA*s represents the island population and nuA*(1-s) represents the mainland population. We enforced an upper limit of 0.5 for s, which constrains the incipient island population at less than 50% of the ancestral population. To determine if intervals of continuous migration or discrete admixture events explain additional features of the 2D-JSFS, we defined models that included discrete admixture events for the vicariance and founder event models, in which a fraction f of the mainland population is instantaneously present in the postadmixture island population. These events were placed immediately after the initial divergence, between two discrete time intervals allowing for genetic drift, or at the end of a single drift interval. Although we included a model of ancient migration and a model of secondary contact for the vicariance scenario, these required intervals of continuous migration, which are less plausible for island systems than discrete admixture events.

Using threefold perturbed random starting parameters, we performed 50 optimization replicates using the Nelder-Mead method (optimize_log_fmin) with a maximum of 20 iterations for each of the models included in the 2D model population comparison sets. Each optimized parameter set was used to simulate the 2D-JSFS, and the log-likelihood of the 2D-JSFS given the model was estimated using the multinomial approach. We used the best scoring replicate of each model to select starting parameters for a second round of 50 replicate optimizations involving twofold parameter perturbations, and the parameter values from the best replicate were subsequently used to generate onefold perturbed starting parameters for a final set of 100 replicate optimizations. All newly created models for our analyses are available at: https://github.com/dportik/dadi_pipeline. We compared the results from our 2D modelling using the Akaike information criterion (AIC), and the replicate with the highest likelihood for each model was used to calculate AIC scores, Δ AIC scores, and Akaike weights (ω_i) -WILEY

(Burnham & Anderson, 2002). We did not transform raw parameters using a mutation rate because our primary aim was to perform model selection, and parameter values should ideally be obtained through bootstrapping to produce confidence intervals (Gutenkunst et al., 2009). We provide the estimate of θ ($\theta = 4N_{ref\mu}L$, where L is the total length of sequenced region SNPs were ascertained from) the effective mutation rate of the reference population, which here corresponds to the ancestral population.

2.6 Morphological and colour pattern variation

Regional differences in both body size and colour pattern are documented throughout the range of *A. paradorsalis* in Cameroon (Amiet, 2009). We collected and analyzed body size data and 14 linear morphological measurements for adult specimens of *A. p. paradorsalis* and *A. p. manengubensis* as described in Appendix S3. We also characterized and examined the phylogenetic distribution of dorsal colouration as described in Appendix S3, and image data are available from: osf.io/ghwam.

3 | RESULTS

3.1 | Phylogeographical structure and divergence dating

We recovered three distinct lineages in our mtDNA dataset that correspond to A. p. manengubensis, northern A. p. paradorsalis (north of the Sanaga River), and southern A. p. paradorsalis (south of the Sanaga River; Figure 2a). The Bioko Island population forms a distinct group nested within northern A. p. paradorsalis and we find strong support for A. p. manengubensis as sister to the northern and Bioko Island clade. The main divergence event between the northern and southern lineages is estimated to have occurred in the Pleistocene at 2.22 Ma (95% highest posterior density region [HPD] 1.53–2.99 Ma), with subsequent divergence between the northern lineage and A. p. manengubensis approximately 1.56 Ma (0.99–2.19, 95% HPD; Table 1). The estimated times at which lineages in the Bioko Island population coalesce is approximately 100 ka (40– 230 ka, 95% HPD; Table 1).

In our DAPC analyses of 3,917 unlinked SNPs, we detected four genetically distinct populations that correspond to A. *p. manengubensis* and three populations of A. *p. paradorsalis* (Figure 2c). We inferred the same three populations of *Afrixalus p. paradorsalis* with our hierarchical maximum likelihood population clustering analyses (Figure 2d). These populations correspond to the northern and southern lineages recovered in the mtDNA analyses and a distinct Bioko Island lineage. Our Bayesian phylogenetic analysis of genomewide SNP variation using a coalescent model recovered the same lineage relationships as the mtDNA analyses (Figure 2b), and with high support for all nodes (>95% posterior probability). Based on a human mutation rate, the divergence between the northern and southern lineages is estimated at 2.68 Ma (2.20–3.15, 95% HPD), and A. *p. manengubensis* diverged from the northern lineage approximately



FIGURE 2 Chronograms of the Afrixalus paradorsalis complex from (a) the BEAST analysis of mtDNA (16s) sequence data calibrated with a 2% per Myr divergence rate, and (b) Bayesian coalescent analysis of 3,917 SNPs using SNAPP. Median ages are provided above nodes, with error bars representing the 95% HPD shown, and nodes with high support are filled black (posterior probability >0.95). Population identification and assignment results from analyses of 3,917 SNPs using (c) a discriminant analysis of principal components of 48 A. *p* paradorsalis (11 northern, 23 southern and 14 Bioko) and two A. *p. manengubensis* and (d) maximum likelihood population clustering of 48 A. *p. paradorsalis* (two samples of A. *p. manengubensis* not shown) using ADMIXTURE. The colour scheme matches the sampling localities depicted in Figure 1

TABLE 1 Summary of divergence date estimates for key nodes in the mitochondrial phylogeography

Northern+Southern	A. p. manengubensis	TMRCA North	North+Bioko	TMRCA Bioko	TMRCA South
2.22 (1.53–2.99)	1.56 (0.99–2.19)	0.41 (0.20–0.69)	0.22 (0.10–0.42)	0.10 (0.04–0.23)	0.77 (0.42–1.21)

Notes. The median date estimates for a node are given in millions of years, along with associated 95% highest posterior density region.

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1.14 Ma (0.89–1.43, 95% HPD). Finally, the northern and Bioko lineages are estimated to have shared a common ancestor 420 ka (330–540 ka, 95% HPD).

3.2 Molecular diversity and spatial connectivity

We found very little diversity in mtDNA or nuDNA in the A. *p. manen-gubensis* lineage; however, this is likely due to our small sample size for A. *p. manengubensis* (Table 2). By contrast, our sampling for the Bioko Island population was on par with that of the northern and southern populations of A. *p. paradorsalis* and yet our estimates of genetic diversity were much lower in the island lineage relative to the continental lineages (Table 2). Estimates of pairwise F_{ST} indicate that the A. *p. manengubensis* lineage is highly divergent from the northern lineage of A. *p. paradorsalis* on the basis of mitochondrial ($F_{ST} = 0.91$) and nuclear sequence data ($F_{ST} = 0.42$; Table S3.1). Within the three A. *p. paradorsalis* lineages, the northern and southern lineages are also well differentiated (mtDNA $F_{ST} = 0.82$, nuDNA $F_{ST} = 0.54$), whereas the Bioko lineage is only moderately differentiated from the northern lineage (mtDNA $F_{ST} = 0.51$, nuDNA $F_{ST} = 0.23$; Table S3.1).

The EEMS analysis highlighted a clear migration barrier along the Sanaga River, starting in northeast Cameroon and following the river diagonally to its mouth at the Bay of Douala (Figure 3a). Likewise, there is a large area of reduced migration in central Gabon that roughly coincides with the Ogooué-Ivindo Rivers. We detected exceptionally high genetic diversity along a substantial portion of the CVL and in Gabon at the intersection of the Ogooué-Ivindo Rivers (Figure 3b), and we found comparatively lower genetic diversity in the Bioko Island lineage.

3.3 Demographic modelling

For the northern and southern pairwise comparison, we found unambiguous support for two refugial models (99.9% of the total model weight) that involve divergence in isolation followed by population size expansion and secondary contact (Table S3.2), The models differ only in whether they include one or two migration rates, and we found stronger support for the model with a single migration rate between the populations (Δ AIC = 3.4, ω_i = 0.844; Figure 4a). The model with two migration rates estimated greater migration from the northern to the southern population (Table S3.2). The initial sizes of the northern and southern populations were comparable, but the northern population became larger than the southern population following the period of demographic expansion. We found the threeepoch refugial models, which involve divergence in isolation, secondary contact, and contemporary isolation, were generally a poor fit (Δ AIC range = 261.2–285.2). These results strongly suggest that after an initial divergence period in isolation, gene flow has resumed between the northern and southern regions.

In our investigation of the northern and Bioko Island populations, we found that models of pure vicariance provided a substantially better fit to our data than the founder event models (Table S3.3). The best overall model consists of vicariance and genetic drift, followed by a late discrete admixture event (ΔAIC = 3.0, ω_i = 0.79; Figure 4b). Across all the vicariance models included in our analyses, the point estimates for the proportion of the ancestral population founding the Bioko Island population ranged from 27.7-36.6% (Table S3.3) and our results demonstrate that Bioko Island likely maintained a stable population size, rather than experiencing exponential growth associated with founder events. In the top-ranked model, we estimated that the fraction of the mainland (northern) population present in the post-admixture island population (f) was quite low (1.5%, Table S3.3). These results indicate that if a postvicariance exchange did occur between the mainland and Bioko, the extent of this exchange was minimal. This notion is further supported by the second-best model, which consists of vicariance without any migration or admixture events ($\omega_i = 0.17$).

3.4 Morphological and colour pattern variation

A summary of all measurements is provided in Table S3.4. For both males and females, most of the total variance in morphology was captured in the first two principal components (66.9% and 88.7% for males and females, respectively; Tables S3.5, S3.6). The first principal component (PC1) loaded heavily on SUL indicating that differences in body size were responsible for most of the variance (Tables S3.5, S3.6). The second principal component (PC2) loaded heavily on characters pertaining to leg morphology (TL, THL, FL; Tables S3.5, S3.6). Both male and female A. p. manengubensis are strongly differentiated from all populations of A. p. paradorsalis in our principal components analysis of morphological variation and in the more extensive SUL dataset (Figure 5).

TABLE 2 Summary statistics for mitochondrial locus (16s) and nuclear SNPs (ddRADseq) collected from the Afrixalus paradorsalis complex

	165				Nuclear SNPs						
	N	N _H	bp	θs	θπ	N	Loci	N _A	Р	H _E	θ_{H}
A. p. paradorsalis south	37	17	495	0.0082	0.0108	23	3,310	1.572	0.576	0.0732	0.0789
A. p. paradorsalis Bioko	15	2	495	0.0000	0.0002	14	3,448	1.163	0.164	0.0477	0.0501
A. p. paradorsalis north	19	8	495	0.0058	0.0041	11	3,109	1.315	0.318	0.0759	0.0821
A. p. manengubensis	2	1	495	0.0000	0.0000	2	2,543	0.835	0.051	0.0211	0.0216

N: number of individuals sampled; N_H: number of haplotypes; bp: sequence length in base pairs; θ_s : genetic diversity based on number of segregating sites; θ_{π} : genetic diversity based on pairwise sequence comparisons; loci: number of loci with <25% missing data within the lineage; P: proportion of polymorphic sites; N_A: allelic richness; H_E: expected heterozygosity; θ_{H} : genetic diversity based on expected homozygosity.



FIGURE 3 Contour maps representing the posterior mean of (a) effective migration surface and (b) effective diversity surface, for 3,917 SNPs collected from 48 samples of *Afrixalus*. *p. paradorsalis* (A. *p. manengubensis* excluded). In (a), blue colours represent areas of high migration, or dispersal corridors, whereas orange regions represent areas of low migration, or dispersal barriers. In (b), white colour indicates areas of lower than expected genetic diversity, and dark purple colouration represents higher levels of genetic diversity. Sampling locality symbols are scaled according to the number of samples in a merged locality



FIGURE 4 Population genetic model comparisons for (a) north (n = 8) and Bioko Island (n = 9), and (b) north (n = 8) and south (n = 16) *Afrixalus. p. paradorsalis* using the two-dimensional joint site frequency spectrum (2D-JSFS) and the filtered ddRADseq SNP dataset (2,205 SNPs). A simplified visual representation of the best-fit model is depicted, along with comparisons of the 2D-JSFS for the data, the model, and resulting residuals



FIGURE 5 Bivariate ordination of first two components from a principal components analysis of 15 linear measurements for male (a) and female (b) Afrixalus p. manengubensis and Afrixalus p. paradorsalis. Variation in body size (snout-urostyle length) from the more extensive sample size of male (c) and female (d) Afrixalus p. manengubensis and Afrixalus p. paradorsalis

Among populations of A. p. paradorsalis, pattern type 1 is more common in the Bioko lineage, with only one out of 15 individuals exhibiting pattern type 2 (Figure S3.1). Pattern types 1 and 2 are common in both the northern and southern populations of A. p. paradorsalis, and occur in the same frequency in A. p. manengubensis, and the distribution of pattern types across the phylogeny revealed no clear relationship with population identity or geography.

4 | DISCUSSION

Sky island diversification along the 4.1 **Cameroonian Volcanic Line**

We found that Afrixalus p. manengubensis, the submontane lineage of the A. paradorsalis complex on Mount Manengouba and the adjacent Bakossi Mountains, is differentiated from lowland rain forest

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populations of the complex (A. p. paradorsalis) in both our mtDNA and nuDNA datasets. Mt. Manengouba formed ~1 Ma (Marzoli et al., 2000), with the oldest geochemical samples from the mountain estimated at 1.55 Ma (Fitton & Dunlop, 1985; Gouhier, Nougier, & Nougier. 1974), and this period of mountain formation roughly coincides with our estimate of 1.56 Ma (0.99-2.19 Ma) for divergence between A. p. manengubensis and A. p. paradorsalis. Four additional anuran species are thought to be endemic to Mt. Manengouba (Cardioglossa manengouba, C. trifasciata, Leptodactylodon erythrogaster, and Phrynobatrachus manengoubensis), and several other amphibians occur exclusively on Mt. Manengouba and adjacent mountains (Mt. Kupe, Mt. Nlonako, Bakossi Mts.) (Amiet, 1975; Blackburn, 2008; Herrmann et al., 2005; Portik et al., 2016; Schmitz, Euskirchen, & Böhme, 1999). Comparative evidence from other taxonomic groups is limited, but elevated genetic diversity or patterns of divergence across the CVL have been documented in plants (Budde, González-Martínez, Hardy, & Heuertz, 2013; Hardy et al., 2013), birds (Smith et al., 2000), and chameleons (Barej et al., 2010). Together, this suggests that endemic diversity in this mountainous region has accumulated in a relatively short period of time-a hypothesis that can be further tested by estimating divergence times of other co-occurring endemics. Divergence between submontane and lowland species may reflect allopatric speciation following the formation of elevational barriers to dispersal during uplift of the Cameroon Volcanic Line (CVL). Alternatively, disruptive selection mirroring environmental variation along the elevational gradient may drive diversification through ecological speciation (Zhen et al., 2017). Unfortunately, due to our small sample size we were unable to include A. p. manengubensis in our demographic modelling approach to differentiate between allopatric and parapatric models of speciation. Dense sampling along ecological and altitudinal gradients across the mountain ranges coupled with demographic models may provide insights into the role of gene flow and disruptive selection in driving divergence between the submontane and lowland lineages of A. paradorsalis.

The significantly smaller mean SUL of both male and female A. p. manengubensis individuals is characteristic of many submontane and montane anurans, including the Mt. Manengouba/Bakossi Mts. endemic P. manengubensis, which is also smaller than its nearest relatives (Zimkus & Gvoždík, 2013). Smaller body size may arise in montane populations due to reduced resource availability and slower growth rates in highland ecosystems (Berven, 1982; Licht, 1975); however, trends in anuran body size with increasing elevation vary with respect to life history and other taxon-specific factors (Ma, Tong, & Lu, 2009). In anurans, differences in male advertisement calls are considered one of the most important premating barriers to reproduction, and the dominant frequency of the call a male produces is inversely correlated with body size (Gingras, Boeckle, Herbst, & Fitch, 2013). Consequently, reduced body size in highland populations may reflect selection for higher frequency calls, potentially as a response to elevation-induced ecological or environmental variation such as differences in ambient noise (Hoskin, James, & Grigg, 2009). Alternatively, because of the strong relationship between body size and dominant frequency, differences in mating signal may arise as a by-product of adaptive changes in body size and may ultimately lead to reproductive isolation. Future studies characterizing the advertisement calls of A. *p. manengubensis* and A. *p. paradorsalis* across habitats (e.g., Kirschel et al., 2011), as well as differences in the acoustic environment, may clarify whether these mechanisms are related to body size differences between montane and lowland populations.

4.2 | Strong support for climatic refugia underlying lineage divergence in lowland tropical forests

The distribution of A. *p. paradorsalis* in Lower Guinea encompasses several major landscape features, including the CVL, Sanaga River, and Ogooué-Ivindo rivers, multiple proposed historical forest refugia (Maley, 1996), and two major forest ecotones related to precipitation and seasonal inversion. We recovered two genetically distinct allopatric populations of *A. p. paradorsalis* in Lower Guinea through model-based and nonparametric clustering methods (northern, southern; Figure 2c,d), and found evidence for decreased effective migration occurring along the putative population boundary that overlaps with the Sanaga River (Figure 3a). We did not find any additional population structuring coinciding with the CVL, Ogooué-Ivindo rivers or forest-specific ecotones, suggesting that these landscape features have not played a significant role in the diversification of *A. p. paradorsalis*.

To determine whether divergence of the northern and southern populations resulted from limited dispersal across the Sanaga River or from climate-induced forest contractions, we assessed demographic models derived from expected population demographic responses to riverine barriers and climatic refugia. Our demographic model selection supported a refugial model involving divergence in isolation, followed by size expansion in both populations accompanied by gene flow (Figure 4a; Table S3.2). Given the results of contemporary gene flow across the Sanaga River, an isolation by river scenario for these lineages is only feasible if major historical changes occurred in course or flow of the Sanaga River surrounding the early Pleistocene divergence time between the lineages. A combination of evidence from offshore fluvial paleodrainage systems (Ngueutchoua & Giresse, 2010) and divergence-dating estimates of endemic freshwater fishes (Day et al., 2013; Goodier, Cotterill, O'Ryan, Skelton, & de Wit, 2011; Pinton, Agnèse, Paugy, & Otero, 2013) indicates that recent changes in the course of the Sanaga are unlikely, and therefore the initial isolation of A. p. paradorsalis populations is better explained by shifts in forest cover that occurred in this time period (Anhuf et al., 2006; Cowling et al., 2008; Maley, 1996; deMenocal, 2004). Our results parallel those of the sympatric Gaboon Forest Frog (Scotobleps gabonicus), including temporal overlap in the forestrefugia-driven divergence of the northern and southern populations across these species (A. p. paradorsalis: 2.22 Ma, 1.53-2.99 Ma 95% HPD; S. gabonicus: 2.97 Ma, 2.61-3.34 Ma 95% HPD; Portik et al., 2017). If forest-refugia continue to emerge as a widespread mechanism of divergence, investigating the synchronicity of divergence times across taxa could statistically demonstrate whether species display a shared response to key climate change events.

4.3 | Marine incursions underlie vicariance and population divergence on Bioko Island

The Bioko population of A. p. paradorsalis is moderately genetically differentiated from the northern population of A. p. paradorsalis in both our mtDNA and SNP datasets, indicating that the island populations are genetically isolated from their mainland counterparts. We recovered a late Pleistocene origin for the monophyletic Bioko lineage (TMRCA 40-230 ka based on mtDNA). This divergence time estimate overlaps with those obtained for the Bioko Island populations of Hyperolius ocellatus (30-290, and 90-460 ka) and H. tuberculatus (40-210 ka), and the three sets of estimates coincide with periods of land bridge connectivity between Bioko and the continent (Bell et al., 2017). Although amphibians have dispersed overseas to colonize the oceanic islands in the Gulf of Guinea archipelago (Bell, Drewes, Channing, et al., 2015; Measey et al., 2007), relatively recent divergence and moderate genetic diversity in Bioko's amphibian populations (relative to those on São Tomé and Príncipe; Bell, Drewes, & Zamudio, 2015) are more consistent with vicariance due to marine incursions than founder events resulting from overseas dispersal (Bell et al., 2017). Furthermore, the best-fit demographic model strongly supports a scenario of vicariance by marine incursion, rather than overseas dispersal (Figure 4b, Table S3.3). Our demographic modelling also revealed support for a discrete admixture pulse, in which a small proportion of the mainland population entered the Bioko Island population in the recent past (comprising ~1.5% of the Bioko population, Table S3.3). Although we found support for an exchange between the island and mainland, it is quite limited in extent, and models involving more extensive island-mainland interactions (such as periods of continuous gene flow) were a poor fit (Table S3.3). The monophyly of mtDNA haplotypes on Bioko Island also supports a pattern of limited exchange, as multiple colonization events would be expected to produce a pattern of paraphyly with the mainland clade (Figure 2a).

Peripatric speciation may occur between island and mainland populations of A. p. paradorsalis if the two lineages undergo substantial divergence due to independent selective pressures or genetic drift prior to the next period of connectivity, leading to reproductive isolation even if sympatry is eventually restored. Alternatively, in the absence of reproductive isolation, introgression may occur during secondary contact, and genomic and phenotypic differentiation may erode as a result. Though we did not find significant morphological differences between island and mainland populations of A. p. paradorsalis, the relative rarity of Pattern Type 2 on Bioko (Figure S3.1) may reflect a difference in allele frequency between these two groups, particularly if dorsal colour pattern follows a single locus mode of inheritance in which each pattern is encoded by a separate allele, as demonstrated in Eleutherodactylus coqui (O'Neill & Beard, 2010). In addition, although Bioko Island and northern A. p. paradorsalis males do not differ in body size and therefore likely produce advertisement calls with similar dominant frequencies, a recent study of advertisement call evolution in island reed frogs indicates that closely related, allopatric species may differ dramatically in the number Journal of Biogeography -WILEY

of pulses a male produces per call (Gilbert & Bell, 2018). Consequently, future efforts to characterize advertisement calls across island and mainland populations of *A. p. paradorsalis* may recover early stages of divergence in this important component of species recognition and mate choice.

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DATA ACCESSIBILITY

We developed a project page (https://osf.io/fvh9k) using the Open Science Framework that includes our ddRADseq haplotypes format 1792

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files (osf.io/nrsy6), and the input data, analysis instructions, and results files for BEAST (osf.io/g54k7), EEMS (osf.io/5dcy2), SNAPP (osf.io/ b7395), ADMIXTURE (osf.io/b8u7 g), and $\delta a \delta i$ (osf.io/63vy7). All newly created demographic models for our analyses are incorporated into an updated version of the model-testing pipeline of Portik et al. (2017), freely available at: https://github.com/dportik/dadi_pipeline. All mitochondrial sequences generated for this project are deposited in GenBank (accession numbers: MH378334-378405).

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BIOSKETCH

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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