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Leap-frog dispersal and mitochondrial introgression: Phylogenomics and biogeography of *Limnonectes* fanged frogs in the Lesser Sundas Archipelago of Wallacea

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Abstract

Aim: The Lesser Sunda Islands are situated between the Sunda and Sahul Shelves, with a linear arrangement that has functioned as a two-way filter for taxa dispersing between the Asian and Australo-Papuan biogeographical realms. Distributional patterns of many terrestrial vertebrates suggest a stepping-stone model of island colonization. Here we investigate the timing and sequence of island colonization in Asian-origin fanged frogs from the volcanic Sunda Arc islands with the goal of testing the stepping-stone model of island colonization.

Location: The Indonesian islands of Java, Lombok, Sumbawa, Flores and Lembata.

Taxon: *Limnonectes dammermani* and *L. kadarsani* (Family: Dicroglossidae)

Methods: Mitochondrial DNA was sequenced from 153 frogs to identify major lineages and to select samples for an exon-capture experiment. We designed probes to capture sequence data from 974 exonic loci (1,235,981 bp) from 48 frogs including the outgroup species, *L. microdiscus*. The resulting data were analysed using phylogenetic, population genetic and biogeographical model testing methods.

Results: The mtDNA phylogeny finds *L. kadarsani* paraphyletic with respect to *L. dammermani*, with a pectinate topology consistent with the stepping-stone model. Phylogenomic analyses of 974 exons recovered the two species as monophyletic sister taxa that diverged ~7.6 Ma with no detectable contemporary gene flow, suggesting introgression of the *L. dammermani* mitochondrion into *L. kadarsani* on Lombok resulting from an isolated ancient hybridization event ~4 Ma. Within *L. kadarsani*, the Lombok lineage diverged first while the Sumbawa and Lembata lineages are nested within a Flores assemblage composed of two parapatrically distributed lineages meeting in central Flores. Biogeographical model comparison found strict stepping-stone dispersal to be less likely than models involving leap-frog dispersal events.

Main conclusions: These results suggest that the currently accepted stepping-stone model of island colonization might not best explain the current patterns of diversity in the archipelago. The high degree of genetic structure, large divergence times, and absent or low levels of migration between lineages suggests that *L. kadarsani* represents five distinct species.

1 | INTRODUCTION

The Lesser Sundas Archipelago, as part of the vast biogeographical filter zone that later came to be known as "Wallacea", played an instrumental role in the formulation of Alfred Russel Wallace's groundbreaking theories on biogeography (Huxley, 1868; Wallace, 1860). Thus, it is perhaps surprising that our understanding of the biogeography of the Lesser Sundas remains quite underdeveloped more than 150 years after Wallace's return from the Indo-Australian Archipelago. The limited progress is primarily attributable to lack of comprehensive taxonomic sampling with corresponding genetic data, as well as the lack of a detailed geological model describing the tectonic history of the archipelago, particularly with respect to the ages of the constituent islands (the length of time that each island has been continuously emergent) and the history of inter-island connectivity. As a result, our current understanding of Lesser Sundas biogeography has been based primarily on analyses of faunal assemblages and species composition patterns (e.g. How & Kitchener, 1997) rather than on more detailed time-calibrated phylogenetic and demographic approaches that offer the potential to glean information from estimates of intraspecific as well as interspecific relationships (e.g. Tänzler et al., 2016). Superficial faunal analyses have suggested that taxa colonized the archipelago via a stepping-stone process (Hisheh, Westerman, & Schmitt, 1998), but this requires empirical confirmation with a more fine-scale approach.

The Lesser Sundas Archipelago represents one of the most geologically active and tectonically complex regions in the world. The archipelago, which formed via subduction in the west and continent-island arc collision in the east (Rigg & Hall, 2011; Spakman & Hall, 2010), contains three geologically distinct components: the Sunda Arc, the Banda Arc and Sumba Island (Figure 1). The Sunda Arc is a volcanic island chain formed by subduction of the Australian plate under the Eurasian plate, and includes (from west to east) Java, Bali, Lombok, Sumbawa, Flores and Lembata. *Limnonectes* fanged frogs occur only on the Sunda Arc islands and we therefore consider only the Sunda Arc in this analysis.

The position and linear arrangement of the Lesser Sunda Islands have allowed the archipelago to act as a two-way filter between the Asian and Australo-Papuan biogeographical realms, with each deep-water barrier between islands effectively limiting the eastward or westward movement of some species. In many taxonomic groups, and in reptiles and amphibians in particular, this filtering effect has left a west-to-east clinal reduction in Asian species and an east-to-west clinal reduction in Australo-Papuan species across the archipelago (Darlington, 1957; Whittaker & Fernández-Palacios, 2007). The simplest mechanism proposed to create such a pattern is a stepping-stone model of island colonization whereby the island nearest to the source is predicted to have been colonized first from the adjacent continental shelf source, followed by the next closest island, and so on until they reach the island furthest from the source. In terms of expected phylogenetic patterns, this colonization scenario will produce a pectinate tree topology where the first lineage to diverge within the Lesser Sundas would represent the island closest to the shelf of origin, and the most recently diverged lineages would represent the most distant islands from the shelf (see Outlaw & Voelker, 2008).
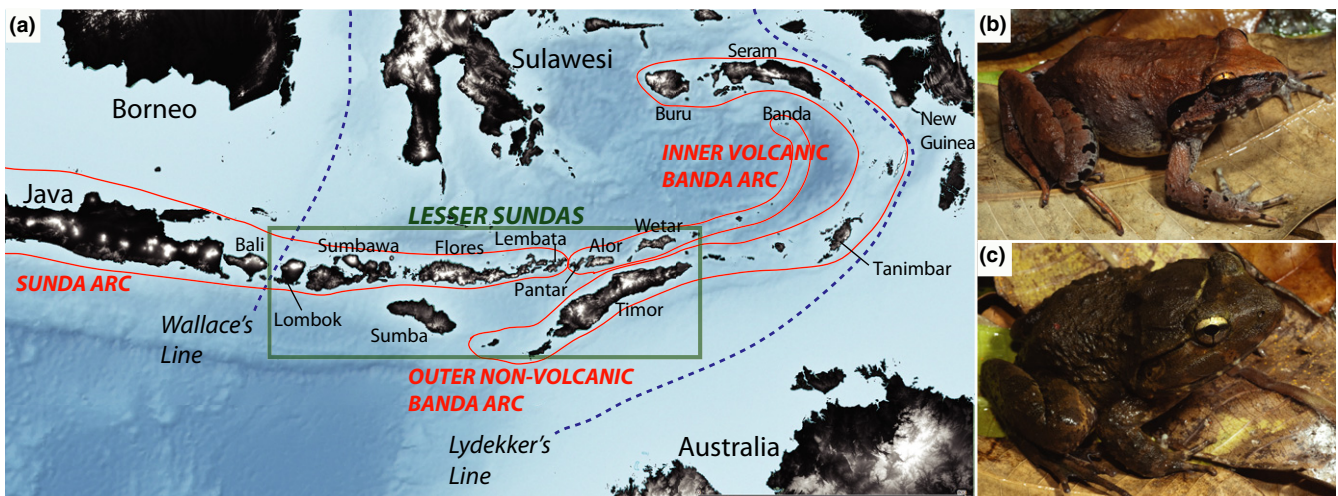

FIGURE 1 (a) Map of southern Wallacea. (b) *Limnonectes dammermani* occurs on Lombok and (c) *L. kadarsani* occurs on Lombok, Sumbawa, Flores, Adonara and Lembata (Photos: J. McGuire) [Colour figure can be viewed at wileyonlinelibrary.com]

The stepping-stone model assumes that all the islands were either present before colonization commenced, or that the islands formed from west-to-east (for which there is limited evidence) or east-to-west (for which there is no evidence). However, the geological history of the region is quite complex and still poorly understood, with different islands becoming continuously emergent at vastly different times in the past with rough estimates ranging from ~2 Myr for the Banda outer arc islands (Timor, Savu, and Rote) and Sumba, ~5 Myr for the Banda inner arc islands, and up to ~11 Myr for the Sunda Arc islands such as Lombok, Sumbawa and Flores (Hall, 2009, 2011; Spakman & Hall, 2010). During glacial maxima, when sea levels were up to 120 m lower than present day, various sets of islands may have become connected by land bridges such as Lombok+Sumbawa, which are separated by a channel depth of ~100 m, and Flores+Lembata, which are separated by a much shallower channel (Bintanja, van de Wal, & Oerlemans, 2005; Voris, 2000). Island age and connectivity certainly influenced the colonization history of species that entered the archipelago, yet there are no published phylogeographical studies of Lesser Sunda taxa that explicitly address these historical biogeographical factors. Here we provide the first biogeographical analysis of a Lesser Sundas focal group that overcomes some of these prior deficiencies by employing the power of rigorous phylogenomic and population genomic analyses. Our study examines a clade of *Limnonectes* fanged frogs that colonized the volcanic Sunda Arc islands east of Bali, which represent the most linearly arranged and stepping-stone like islands of the Lesser Sundas. We thus test the stepping-stone model in a system most likely to have experienced a stepping-stone colonization process.

Many of the larger islands in Wallacea are inhabited by anurans that have arrived by overwater dispersal, although some commensal species have been introduced by humans (Reilly et al., 2017). The Lesser Sunda Islands contain 18 species of anurans including two species of fanged frogs of the genus *Limnonectes* (Family Dicroglossidae), which are common throughout Southeast Asia (Inger, 1999). Both species are restricted to the Sunda Arc Islands (Figure 1), with *L. dammermani* (Mertens, 1929) reported from Lombok (with unconfirmed reports from Sumbawa and Flores) and *Limnonectes kadarsani* reported from Lombok, Sumbawa, Flores, Adonara and Lembata (Iskandar, Boeadi, & Sancoyo, 1996; Iskandar & Mumpuni, 2004a,b). Research on *Limnonectes* from other parts of Southeast Asia, such as the Greater Sunda Islands, Sulawesi and the Philippines have shown that these frogs are not only able to colonize oceanic islands but that they also have frequently diverged ecologically and morphologically, radiating into new species that can be sympatrically distributed (Evans et al., 2003; Iskandar, Evans, & McGuire, 2014; McLeod, 2010; Setiadi et al., 2011). The two species of *Limnonectes* in the Lesser Sundas are most closely related to *Limnonectes microdiscus* of the adjacent Sunda Arc islands of Java and Sumatra, and thus have an Asian origin (Evans et al., 2003). The presence of two *Limnonectes* species on the island of Lombok suggests that species formation either occurred via allopatric divergence followed by a second dispersal event to Lombok, or by way of in situ diversification, a process which few have proposed to account for the species-level diversity in the Lesser Sundas.

In this study, we employ both mitochondrial and genomic datasets for Lesser Sundas *Limnonectes*, utilizing phylogenomic, population genetic and biogeographical model testing analyses to address three main questions: (a) did in situ speciation occur on Lombok, (b) is island colonization within *L. kadarsani* consistent with the stepping-stone model and (c) does island connectivity during glacial maxima help explain the pattern and levels of divergence between *L. kadarsani* populations.

2 | MATERIALS AND METHODS

2.1 | Sampling

Limnonectes specimens were collected from the field, including the outgroup *L. microdiscus* from Java, as well as *L. dammermani* from Lombok and *L. kadarsani* from Lombok, Sumbawa, Flores and Lembata islands (see Appendix S3). The Lesser Sundas samples and tissues were collected on expeditions to the Lesser Sunda Islands that occurred during 2010 and 2011. Liver tissue was dissected from euthanized frogs and either stored in RNALater, or flash frozen in liquid nitrogen.

2.2 | Mitochondrial DNA data analyses

The *16S* ribosomal RNA gene was sequenced for 153 frogs from the Lesser Sunda Islands and for three *L. microdiscus* from Java (Table S3.1). MtDNA laboratory methods can be found in the Appendix S1. The sequence alignment of 874 bp was imported into JMODELTEST 2.1.4 (Darriba, Taboada, Doallo, & Posada, 2012) to determine the best-fit model of sequence evolution (HKY+Γ). A run using BEAST 2.4.8 (Bouckaert et al., 2014) was conducted using the HKY+Γ nucleotide substitution model, a Birth-Death tree prior and a strict molecular clock model with a clock rate of 0.0065. This clock rate corresponds to 1.3% sequence divergence per million years which is an empirically derived estimate for the *16S* gene from the ranoid frog, *Rana boylii* (Macey et al., 2001). A preliminary run was carried out to determine the appropriate number of generations required to achieve ESS values for each parameter that were >200, as viewed in TRACER 1.6 (Rambaut & Drummond, 2009). Once the appropriate run length was determined, two separate runs of 100 million generations were carried out, sampling every 10,000 generations for a total of 10,000 saved generations per run. A burnin of 10% was removed from each of the two runs and the remaining 18,000 trees were combined to create a 50% majority rule consensus tree. The tree was rooted using the outgroup *L. microdiscus* from Java. Nodal support was assessed using posterior probability values. A maximum likelihood tree was also estimated using the program RAXML 8.1.15 (Stamatakis, 2014). The GTRCAT model of sequence evolution was applied, and nodal support was assessed with 1,000 bootstrap replicates.

2.3 | Exon-capture

We obtained genomic data for Lesser Sundas *Limnonectes* using transcriptome-based exon-capture (Bi et al., 2012, 2013; Blom, Bragg, Potter, & Moritz, 2017; Jones & Good, 2016), a recently developed method that allows for the rapid and inexpensive collection of massively multilocus sequence data from non-model organisms. Transcriptome-based exon-capture methods provide datasets ideal for phylogenetic and demographic inference as they can screen hundreds to thousands of independent loci that can be quite long (>1,000 bp) with roughly equal capture efficiency across samples characterized by high levels of nuclear sequence divergence (Bragg, Potter, Bi, & Moritz, 2016; McCartney-Melstad, Mount, & Shaffer, 2016; Portik, Smith, & Bi, 2016). Additionally, the exon-capture method returns a large amount of non-coding intron and flanking sequence data suitable for historical demographic analyses that require neutrally evolving loci (Luikart, England, Tallmon, Jordan, & Taberlet, 2003).

Our capture design utilized orthologous exonic targets derived from two transcriptomes of *L. kadarsani* (Table S3.2). These exon targets were used to design probes for in-solution capture reactions (MYbaits) to screen 48 samples (chosen based on mtDNA results) for 974 independently evolving genes. Laboratory methods for library preparation, capture reactions and bioinformatic pipeline can be found in the Appendix S1.

2.4 | Phylogenomic analyses

The concatenated alignment (1,235,981 bp) of all sequence data (Target+Flanking) was subjected to an unpartitioned maximum likelihood phylogenetic analysis using RAXML (Stamatakis, 2014) under the GTRCAT model of sequence evolution. Nodal support was assessed with 1,000 nonparametric bootstrap replicates.

Individual gene trees for each of the 974 genes were estimated using RAXML to serve as input files for a summary multispecies coalescent species tree approach implemented in ASTRAL-II (Mirarab et al., 2014). For this analysis, each individual was treated as a "species" because it was unclear where the species boundaries lie within this system, thus allowing for direct comparisons with the topology of the concatenated RAXML tree.

2.5 | Population structure of *L. kadarsani*

A principal component analysis (PCA) was conducted by extracting all SNPs from *L. kadarsani* individuals using the SMARTPCA component of EIGENSOFT 6.1.4 (Patterson, Price, & Reich, 2006) to get a covariance matrix of the genotypic data, which was plotted in R.

All informative SNPs (27,941 total) from the 974 genes for the 36 *L. kadarsani* individuals were used to create an input file for the program STRUCTURE (Pritchard, Stephens, & Donnelly, 2000). The program was run under the linkage model for 25,000 generations as burnin, followed by 100,000 generations for $K = 1$ through $K = 8$ populations with 10 replicates per K. The results were then imported into STRUCTURE HARVESTER (Earl, 2012) to determine the most likely number of populations as determined by both the Delta K method and highest mean estimate of the Ln probability of the data. The program was then run for 1,000,000 generations (100,000 generations as burnin) for the most likely number of K as determined by the two methods mentioned above.

2.6 | Inter-island demographics

We performed a series of demographic analyses to assess effective population sizes of extant and ancestral populations, timing of divergences and rates of migration between putatively distinct lineages of *L. kadarsani*. These analyses were undertaken using the program G-PHOCS (Gronau, Hubisz, Gulko, Danko, & Siepel, 2011), which is an isolation-with-migration program that is capable of dealing with unphased genomic sequence data from unlinked neutrally evolving loci. For these analyses, we utilized flanking sequences from each locus because these regions are presumably neutral and likely are similarly influenced by the evolutionary history and demography of *Limnonectes* populations (Luikart et al., 2003). After filtering, 659 flanking region (non-coding) loci were retained for analysis. Each population comparison was run for 500,000–1,000,000 generations discarding 10% as burnin after visually checking the parameter traces for convergence. Run outputs were visualized in TRACER (Rambaut & Drummond, 2009) to assess the posterior distributions of the demographic parameters.

A nuclear DNA mutation rate of 1.42×10^{-9} substitutions per site per year estimated for the ranoid frog genus *Leptopelis* (Allio, Donega, Galtier, & Nabholz, 2017) was used to convert parameter estimates into real world values such as population divergence time in years, effective population size in individuals and population migration rate in individual migrants per generation. All values for Theta and Tau generated by G-PHOCS are scaled by 10^{-4}. Demographic parameter estimates were converted to estimates of effective population sizes (individuals) by dividing the scaled Theta estimate by the mutation rate, then dividing that value by 4. The population divergence time in years was calculated by dividing the scaled Tau estimate by the mutation rate. Migration rate estimates were converted to migrants per generation ($2Nm$) by multiplying the migration estimate by the converted effective population size estimate for the population receiving the gene flow, then dividing that value by the number of generations that have passed (in years) since divergence. A generation time of 1 year was used for migration estimates. The converted 95% HDP low and 95% HDP high estimates for these parameters were calculated in the same manner.

2.7 | Biogeographical model comparison

We used BIOGEOBEARS (Matzke, 2013) to compare dispersal models across the Lesser Sundas for each major island clade. We pruned the phylogenomic species tree to one member of each island clade using the 'Ape' package (Paradis, Claude, & Strimmer, 2004) and adjusted the branch lengths using the *chronos* command with lambda = 1 to make an ultrametric tree. We initially compared the DEC (dispersal,

extinction, cladogenesis) and DEC+J (dispersal, extinction, cladogenesis, plus jump dispersal) models for the stepping-stone dispersal multiplier matrix (i.e. only dispersal between adjacent islands), and the likelihood ratio test significantly favoured the DEC+J model. Thus, we used the DEC+J model to run the analysis five times with each of five dispersal multiplier matrices: (a) stepping-stone; (b) stepping-stone+one leap-frog dispersal event (i.e. dispersal to an island two islands over); (c) stepping-stone+up to two leap-frog events; (d) stepping-stone+up to three leap-frog events; (e) stepping-stone+up to four leap-frog events (i.e. allowing a single dispersal from Bali/Java all the way to Lembata). In the dispersal multiplier matrices, we specified a value of 1 for permitted dispersals and a value of 0.00001 for all others. We generated a distance matrix based on distances calculated to the nearest points between islands using GOOGLE EARTH (Google, 2013), and scaled the distances based on the minimum channel distance between Lombok and Sumbawa. In the analysis, we treated East Flores and West Flores as separate regions as they were well differentiated in the molecular analyses. We compared likelihoods for the different dispersal models using the AIC test implemented in BIOGEOBEARS.

3 | RESULTS

3.1 | MtDNA phylogeny

Both the maximum likelihood and Bayesian phylogenetic analyses of the mtDNA data produced similar topologies when rooted with *L. microdiscus* from Java (Figure 2, Appendix S2). Both phylogenies contain a basal split between all samples from Lombok versus those from Sumbawa, Flores and Lembata. This branching arrangement

suggests that *L. kadarsani* is paraphyletic, with *L. kadarsani* from Lombok more closely related to *L. dammermani* from Lombok than to other *L. kadarsani* populations. The relationships among the Sumbawa, Flores and Lembata populations of *L. kadarsani* indicate that Sumbawa is sister to populations from Flores+Lembata. The major difference between the two phylogenies is that the ML tree suggests that populations on Flores form a monophyletic group that is nested within a Lembata clade (though not well-supported), whereas the Bayesian tree places Lembata as a monophyletic assemblage nested within a Flores clade. Thus, the Bayesian phylogeny is consistent with the stepping-stone model, while the ML tree is not. The Bayesian tree (Figure 2) is time-calibrated and indicates the split between the Lesser Sundas clade and *L. microdiscus* to be ~18.19 Ma (95% posterior density low = 12.7, high = 24.4). The split between all Lombok samples and the rest of the Lesser Sundas was estimated to be ~6.93 Ma (95% posterior density low = 5.1, high = 8.9). The Lombok populations of *L. dammermani* and *L. kadarsani* are estimated to have diverged from one another ~4.16 Ma (95% posterior density low = 2.9, high = 5.6), while the rest of the Lesser Sundas (Sumbawa+Flores+Lembata) populations began diverging from one another ~1 Ma (95% posterior density low = 0.6, high = 1.5). West Flores split from East Flores+Lembata ~0.58 Ma (95% posterior density low = 0.3, high = 0.9), and East Flores split from Lembata ~0.25 Ma (95% posterior density low = 0.1, high = 0.4).

3.2 | Exon-capture data characteristics

After filtering, the total alignment of both the targeted and flanking regions from the 974 genes was 1,235,981 bp. The average
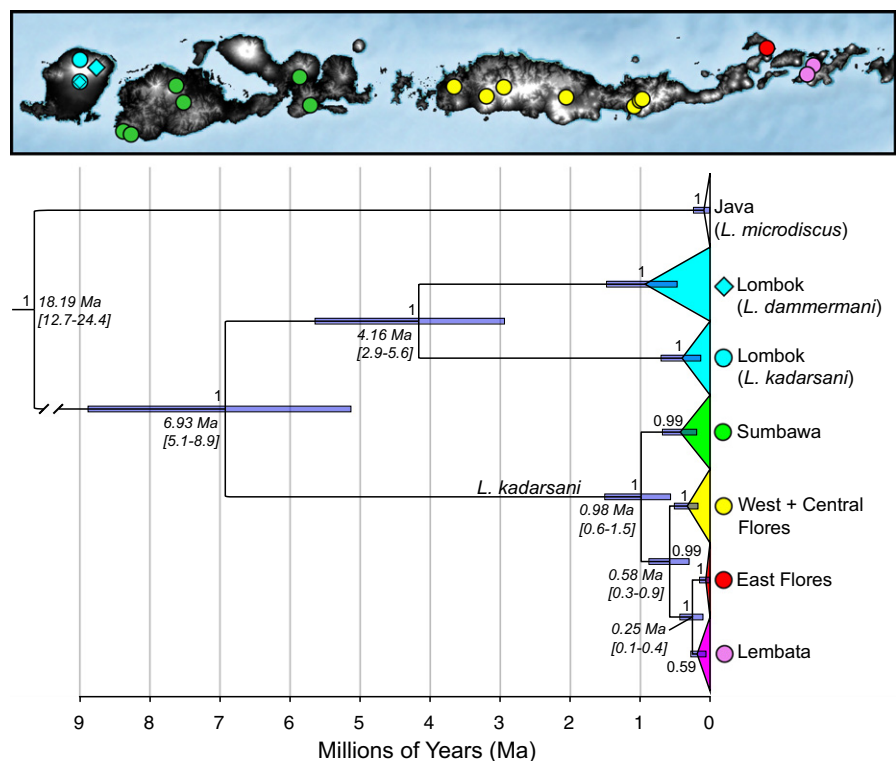

FIGURE 2 Time-calibrated Bayesian phylogeny of the *16S* mitochondrial gene for *Limnonectes microdiscus*, *L. dammermani* and *L. kadarsani* with localities for samples marked on the map above. Bars at nodes represent the 95% confidence intervals for node ages, numbers below nodes are node ages with 95% CI intervals in brackets and numbers above nodes represent posterior probability support. Clade width is not representative of sample size and is collapsed to highlight relationships and timing of splits between clades [Colour figure can be viewed at wileyonlinelibrary.com]

coverage was ~50X for the targeted regions and ~25X for the flanking regions. However, the average coverage for each individual library was highly variable for both the targeted and flanking regions (Figure S2.2).

The average number of taxa per alignment was 47 out of the 48 samples (Figure S2.3c, Figure S2.4a). The number of informative sites has a relatively linear relationship with locus length (Figure S2.3a), and there is on average 7% informative sites per alignment (Figure S2.4c). There is no clear relationship between the alignment length and the percentage of gaps in each alignment (Figure S2.3b). The final length of the alignments ranged from 100 bp up to ~4,500 bp (Figure S2.4b), and the per cent missing data (including missing individual sequences or missing sites) was no higher than 25% for any individual alignment (Figure S2.4d).

3.3 | Phylogenomic analyses

Both the concatenated maximum likelihood tree (Figure 3) and the coalescent-based ASTRAL species tree (Figure S2.5) converged on the same topology with respect to the relationships between major lineages. All major lineages, as well as the major nodes in the tree are well-supported with bootstrap support of 100 (Figure 3). In contrast with the mtDNA phylogenetic estimate, both *Limnonectes dammermani* and *L. kadarsani* were found to be monophyletic sister taxa. Within *L. kadarsani*, samples from Lombok are sister to the rest of the Lesser Sundas. Within the remaining clade, East Flores is sister to a clade composed of samples from Sumbawa, West Flores and Lembata. Finally, the Sumbawa clade is sister to a West Flores + Lembata clade with Lembata nested within West Flores.

3.4 | Population structure within *L. kadarsani*

The PCA of genetic covariance was best explained by the top three components (PC1 = 0.17, PC2 = 0.12, PC3 = 0.08). A plot of PC1 and PC2 returns individuals clustered in groups corresponding to the major phylogenomic clades (Figure 4a). PC1 primarily separates the Lombok individuals from all other populations, while PC2 primarily
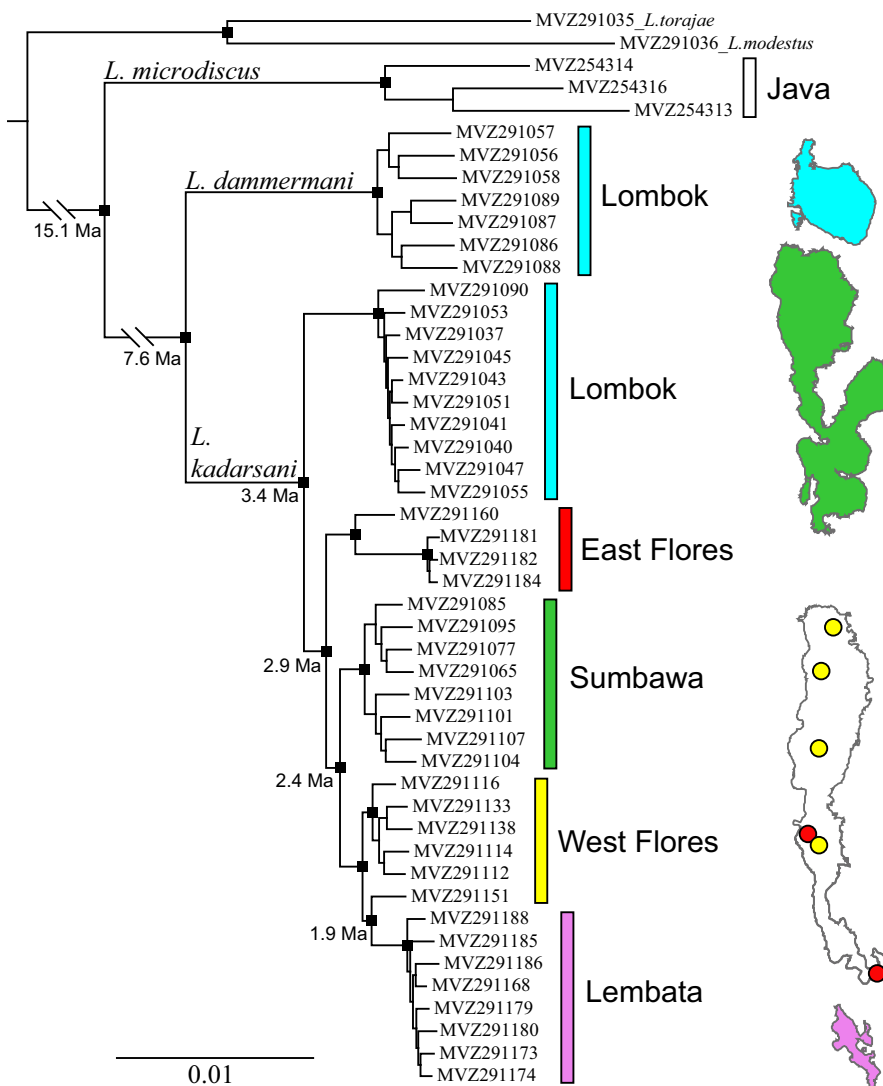

FIGURE 3 Maximum likelihood tree of *Limnonectes* spp produced by RAXML based on the concatenated exon-capture dataset. Black boxes at major nodes represent bootstrap support of 100. Numbers at relevant nodes represent average divergence estimates from demographic analyses [Colour figure can be viewed at wileyonlinelibrary.com]
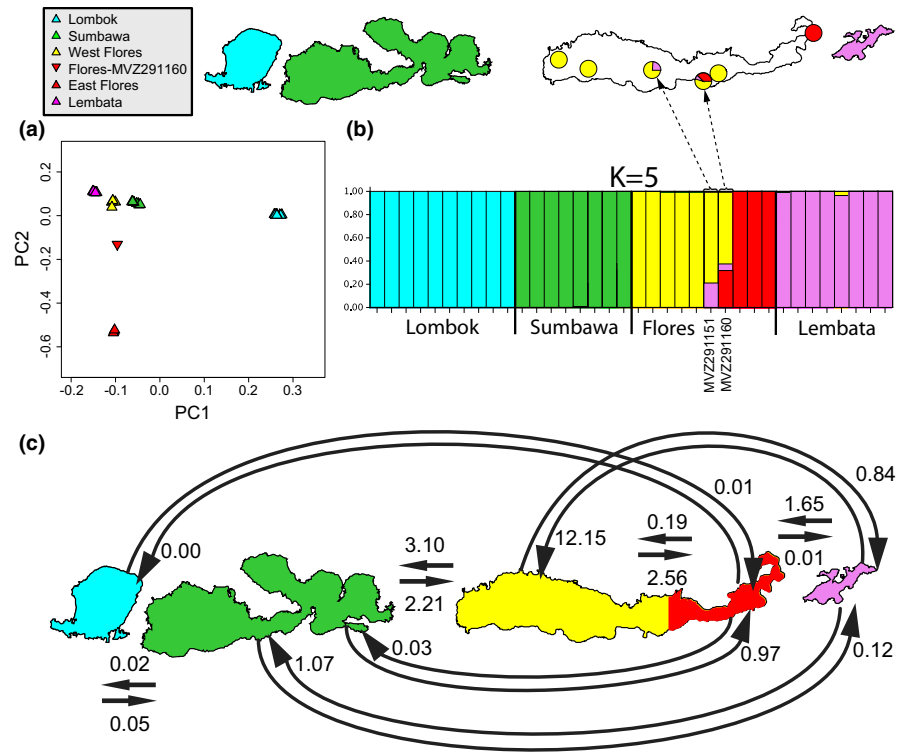
FIGURE 4 Principal component analysis of genetic covariance, genetic clustering analyses and migration estimates for *Limnonectes kadarsani* lineages. (a) PC1 plotted against PC2. (b) Population structure bar plot for an estimated five populations. Each bar represents one individual frog, and the proportion of each colour represents the percent of ancestry. (c) Estimates of migrants per generation between lineages [Colour figure can be viewed at wileyonlinelibrary.com]

separates the East Flores individuals from all others. PC3 was able to separate the Sumbawa and Lembata groups from all other individual (Figure S2.6).

Following the STRUCTURE analysis, the Delta *K* procedure found that the most likely number of populations within *L. kadarsani* is five (Table S3.3). Additionally, the highest mean estimate of the Ln probability of the data also is at *K* = 5 (Table S3.3). The population structure bar-plots show a high level of genetic structuring from *K* = 2 up to *K* = 5, and analyses with larger *K* values still returned only five meaningful clusters corresponding to the results of the *K* = 5 analysis. The *K* = 5 analysis separates all major lineages recovered in our phylogenomic analyses and identifies two apparently admixed *L. kadarsani* individuals (MVZ 291151, MVZ 291160) from central Flores that contain genes from West Flores, as well as from Lembata and/or East Flores (Figure 4b).

3.5 | Demographic analyses

For all G-PHOCS analyses, parameter ESS values were >200 suggesting adequate sampling of parameter space. Converted parameter estimates can be found in Table S3.4. For the purposes of this study, we are interested in divergence times (shown in Figure 3 as the average age for nodes with more than one estimate) and migration (Figure 4c). *Limnonectes microdiscus* diverged from Lesser Sundas species approximately 15.1 Ma (collective 95% CI 13.2–16.8 Ma), followed by a split between *L. dammermani* and *L. kadarsani* approximately 7.6 Ma (95% CI 7.3–7.8 Ma). Within *L. kadarsani* the first divergence is between Lombok and its sister clade ~3.4 Ma (collective 95% CI 3.1–3.7 Ma), then East Flores diverges at ~2.9 Ma

(collective 95% CI 2.6–3.1 Ma), followed by Sumbawa at ~2.4 Ma (collective 95% CI 2.2–2.6 Ma), and finally Lembata and West Flores split at ~1.9 Ma (95% CI 1.8–1.9 Ma).

Migration estimates can be found in Figure 4c. Since their divergence, there is no signature in the nuclear genome of migration between *L. dammermani* and *L. kadarsani* on Lombok, and effectively no migration ($2Nm$ = 0–0.05) between *L. kadarsani* on Lombok and other *L. kadarsani* populations. Sumbawa has limited connectivity with West Flores ($2Nm$ = 2.21 S->WF, $2Nm$ = 3.10 WF->S), East Flores ($2Nm$ = 0.03 EF->S, $2Nm$ = 0.97 S->EF) and Lembata ($2Nm$ = 1.07 L->S, $2Nm$ = 0.12 S->L). Migration is inferred to have been mostly unidirectional between the parapatrically distributed West Flores and East Flores, with approximately 13 times greater migration from West to East ($2Nm$ = 2.6) than from East to West ($2Nm$ = 0.19). Unidirectional migration is also estimated between East Flores and Lembata ($2Nm$ = 0.01 EF->L, $2Nm$ = 1.65 L->EF), and between West Flores and Lembata ($2Nm$ = 0.84 WF->L, $2Nm$ = 12.15 L->WF).

3.6 | Biogeographical model comparison

Model comparison statistics can be found in Table 1. AIC scores were higher for models that allowed for stepping-stone plus one to four leap-frog dispersal events (model 2 AIC = 27.52; model 3 AIC = 27.78; model 4 AIC = 28.92; model 5 AIC = 29.43) versus the model that only allowed stepping-stone dispersals (model 1 AIC = 39.25). The AIC weight is highest for model 2 (AIC_wt = 0.36) and model 3 (AIC_wt = 0.32), with a large drop off for models 4 (AIC wt = 0.18) and 5 (AIC wt = 0.14); thus, we only consider

TABLE 1 Model comparison values from BIOGEOBEARS analyses for colonization of the Lesser Sundas by *L. kadarsani*

BioGeoBEARS model	LnL	numparams	d	e	j	AIC	AIC_wt
1_steppingstone	−16.62	3	1.00E-12	1.00E-12	0.9	39.25	0.001
2_steppingstone+1 leapfrog	−10.76	3	1.00E-12	1.00E-12	0.66	27.52	0.36
3_steppingstone+2 leapfrog	−10.89	3	1.00E-12	0.0006	0.64	27.78	0.32
4_steppingstone+3 leapfrog	−11.46	3	1.00E-12	0.0037	0.51	28.92	0.18
5_steppingstone+4 leapfrog	−11.71	3	1.00E-12	3.80E-06	0.39	29.43	0.14

models 2 and 3 hereafter. Model 2, which allowed for stepping-stone dispersals as well as leap-frog dispersal events bypassing up to one island had the highest likelihood and was most strongly favoured by AIC score. Ancestral range estimation was consistent among dispersal models in placing the ancestor of *L. dammermani* and *L. kadarsani* as most likely on Lombok, but other models did not strongly favour one island over another (Figure 5). The best-supported model, model 2, infers an initial in situ split of *L. dammermani* and *L. kadarsani* on Lombok before *L. kadarsani* dispersed to Sumbawa, followed by a leap-frog dispersal from Sumbawa to East Flores, with a secondary colonization of West Flores from Sumbawa occurring later, and a final leap-frog dispersal from West Flores to Lembata. Model 3, which is only slightly less supported than model 2, differs by having a two-region leap-frog dispersal from Lombok to East Flores followed by a backward leap-frog dispersal from East Flores to Sumbawa, then dispersal from Sumbawa to West Flores, and finally a leap-frog dispersal from West Flores to Lembata.

4 | DISCUSSION

4.1 | Divergence of *L. dammermani* and *L. kadarsani*, and mtDNA introgression

Our phylogenomic analyses confirm the Evans et al. (2003) finding that *Limnonectes dammermani* and *L. kadarsani* are sister taxa. Interestingly, *L. dammermani* is restricted to Lombok Island, whereas *L. kadarsani* has a more expansive range extending from Lombok to Lembata. This distribution suggests the intriguing possibility that *L. dammermani* and *L. kadarsani* initially speciated and diversified on Lombok. Heaney, Kyriazis, Balete, Steppan, and Rickart (2018) found that the smallest island on which any tetrapod has been shown to have speciated is Puerto Rico (for both frogs and lizards), which at 9,104 km² is just greater than twice the size of Lombok (4,514 km²). Thus, if these two species of *Limnonectes* truly diverged on Lombok, this island becomes the smallest for which any tetrapod speciation event has been documented. That said, given that speciation is
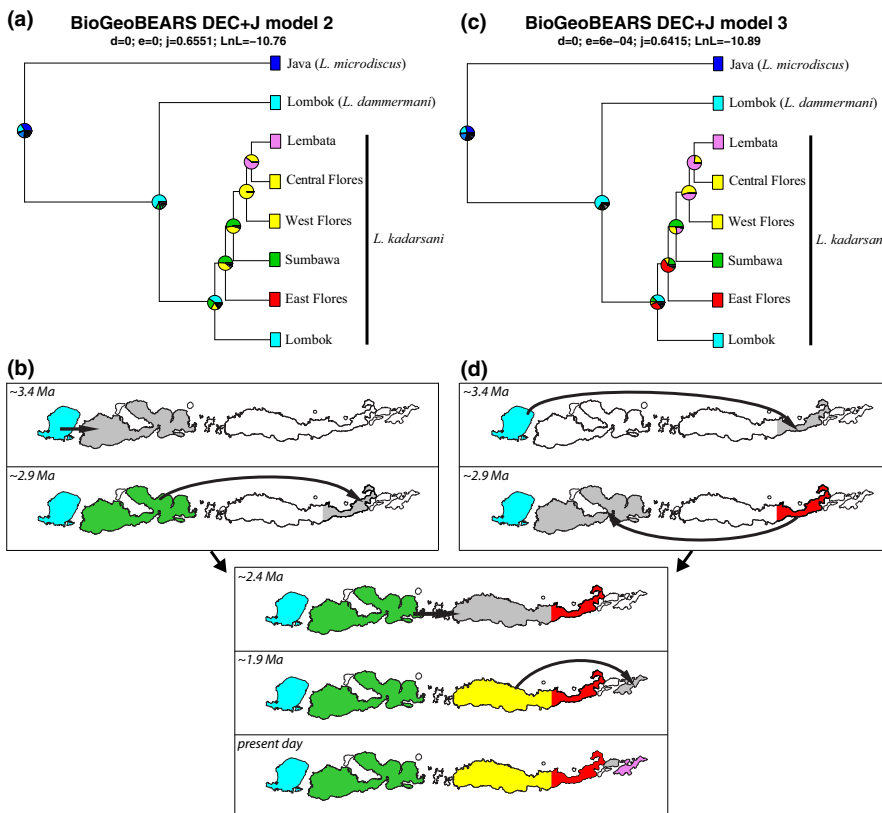

FIGURE 5 Ancestral range reconstructions from BIOGEOBEARS biogeographical model comparisons for the (a) stepping-stone+one island leap model, and the (c) stepping-stone+two island leap model. Map depictions of dispersal events within *Limnonectes kadarsani* for the (b) stepping-stone+one island leap model and the (d) stepping-stone+two island leap model. Pie charts at phylogeny nodes indicate the probability of the ancestral range occupied by that ancestor [Colour figure can be viewed at wileyonlinelibrary.com]

unlikely on such a tiny island as Lombok, an alternative that might be more plausible (even though it would require two dispersal events to achieve) is that the initial split occurred via colonization of Sumbawa from Lombok ~7 Ma as inferred via our population genomic analyses. This hypothesis requires that, following some period of divergence in allopatry, *L. kadarsani* recolonized Lombok. Such a process may have involved several million years of allopatric divergence, during which *L. kadarsani* and *L. dammermani* diverged substantially in body size (our largest *L. kadarsani* sample from Lombok is more than 6X larger [144.7 g] than the largest *L. dammermani* specimen that we collected [24.0 g]). Of course, character displacement following recolonization may also explain this body size variation.

Our mitochondrial analyses, which provide clear evidence of ancient introgression of the *L. dammermani* mitochondrion into *L. kadarsani* on the order of 3–5 Ma, may correspond to the timing of recolonization of Lombok from Sumbawa, which was estimated at ~3.2 Ma based on nuclear DNA. Population demographic analyses with 2Nm migration values well below 1 indicate that no further admixture has occurred between *L. kadarsani* and *L. dammermani*. Additionally, the East Flores lineage is highly distinct in nuclear DNA with demographic analyses suggesting it split from Sumbawa+West Flores+Lembata populations nearly 3 Ma, yet it is minimally divergent in mtDNA from Lembata with an estimated mitochondrial divergence of only 0.25 Ma. These results suggest that mitochondrial introgression has likely also occurred from Lembata into East Flores. Ancient mitochondrial capture events have been documented in many other studies (see McGuire et al., 2007; Toews & Brelsford, 2012) and can now be confirmed for *Limnonectes* fanged frogs.

4.2 | Taxonomic implications

Our robustly supported phylogenomic tree and population clustering analyses suggest that *L. kadarsani* actually represents at least five distinct evolutionary lineages, each of which has been separated from the other lineages for between 1.9 and 3.4 Ma. In the context of the general lineage concept of species (de Queiroz, 1998, 2007), each of these deeply divergent lineages corresponds to a separately evolving species on its own evolutionary trajectory. Their status as independent lineages is supported by our population genomic analyses indicating

strong barriers to gene flow as well as our phylogenomic analyses indicating deep divergence and lineage monophyly. We refer to these lineages as *L. kadarsani* species A-E (Lombok = species A, Sumbawa = species B, West+Central Flores = species C, East Flores = species D and Lembata = species E). Because the type locality of *L. kadarsani* is on Lombok (Iskandar et al., 1996), *L. kadarsani* species B-E will require new names should these lineages be formally described (which we will not pursue here). That *L. kadarsani* is composed of multiple independently evolving lineages is consistent with the idea that species diversity in the Lesser Sundas is substantially underestimated, and that the western Lesser Sundas is an area of endemism representing a distinct Wallacean biogeographical unit (Michaux, 2010).

4.3 | Stepping-stone versus leap-frog dispersal

While Bayesian analysis of our mitochondrial data produced a pectinate topology consistent with a stepping-stone model of colonization, the phylogenomic topology and population divergence estimates uncovered multiple mtDNA introgression events responsible for that topology. While stepping-stone dispersal by a terrestrial vertebrate has been convincingly shown in another linearly arranged southeast Asian archipelago (Yang, Komaki, Brown, & Lin, 2018), our biogeographical analyses reveal that a stepping-stone process can account for some but not all colonization events in this system. For *L. kadarsani*, our best-fitting model included an initial dispersal event from Lombok to Sumbawa ~3.4 Ma, followed by a long-distance leap-frog dispersal from Sumbawa to East Flores ~2.9 Ma. A second bout involved a stepping-stone dispersal event again from Sumbawa but this time to West Flores ~2.5 Ma followed by a second leap-frog dispersal from West Flores to Lembata (bypassing East Flores) ~1.9 Ma.

An interesting finding revealed by the best-fit model shows that all dispersals occurred from west-to-east, a finding that could be explained by the prevailing west-to-east ocean current that runs along the northern side of the volcanic islands (Godfrey, 1996; Gordon & Fine, 1996). However, in straits between islands there is a southward current, and on the southern side of the volcanic islands there is a northeast-to-southwest current (Figure 6). If the relevant historical currents were similar to present day currents, it suggests that frogs that began their rafting journey within straits or from

FIGURE 6 Oceanographic map of the Lesser Sundas showing the major ocean currents as solid blue lines and minor currents as dashed lines (adapted from Godfrey, 1996). Depths of 120 m or less are shaded purple to delimit areas that become emerged (land-positive) during glacial maxima. (1) Java, (2) Bali, (3) Lombok, (4) Sumbawa, (5) Flores, (6) Adonara, (7) Lembata, (8) Pantar, (9) Alor, (10) Wetar, (11) Timor, (12) Rote, (13) Savu and (14) Sumba [Colour figure can be viewed at wileyonlinelibrary.com]
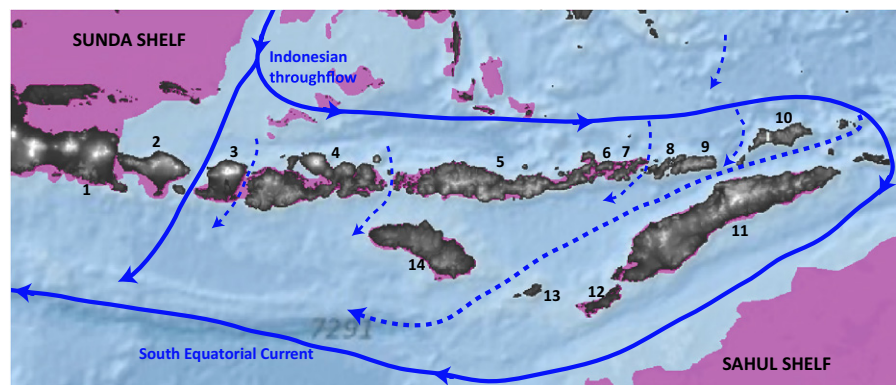
southern shores were likely to have been swept into the Indian Ocean, whereas frogs that dispersed via northern shores would have likely been swept eastward and given an improved chance for successful colonization. For the last 10 million years or more, the currents have flowed from the western Pacific Ocean, through the Indonesian Archipelago, and into the Indian Ocean (a route known as the Indonesian Throughflow; Cane & Molnar, 2001). Based on models of the historical geology and oceanography of the region, it is predicted that as long as the eastern portion of the Sunda Arc has existed (~5 Ma), there has been prevailing southward currents through the straits separating islands (e.g. the Lombok Strait between Bali and Lombok), eastward currents along the north side of the Lesser Sundas and westward currents on the south side of the Lesser Sundas (Holbourn et al., 2005; Kuhnt, Holbourn, Hall, Zuvela, & Käse, 2004). However, monsoonal wind patterns have strongly influenced surface currents for the last 3–4 Ma, and southeast monsoon winds (which generally move surface waters in a western direction) could hypothetically transport rafting frogs from east to west (Spooner, Barrows, De Deckker, & Paterne, 2005).

4.4 | Impact of historical island connectivity

Given the channel depths separating islands in the archipelago and sea level fluctuations during glacial maxima that reduced sea levels by 120–130 m, it is perhaps surprising that we see deep divergences between *L. kadarsani* populations on islands separated by channels <120 m in depth (see Figure 6). For example, Lombok and Sumbawa Islands have been referred to jointly as "Greater Sumbawa" by some workers (Heaney, 1991) because the channel depth between them is only ~100 m, yet *L. kadarsani* on these islands are highly divergent. Similarly, channel depths between East Flores and Lembata are less than 100 m, but their *L. kadarsani* have had minimal connectivity for the last ~2.8 Myr. How can we best explain these findings? One possibility is that the low-lying habitat that connects the islands remains a barrier and is unsuitable for these mountain-dwelling frogs that are dependent on cold, fast-flowing streams. Another possibility is that these channels have become shallower over time by processes such as erosion fill, uplift or volcanic activity. Either way, the presence of old lineages on islands such as Lombok and Sumbawa, which have each experienced cataclysmic volcanic eruptions in the recent past, suggests that these frogs can persist in lowland refugia after massive eruptions (Lavigne et al., 2013; Stothers, 1984).

If we assume that the divergence dates from our genomic data are accurate, then the only colonization events that occurred during the Quaternary ice age are the dispersals from Sumbawa to West Flores and from West Flores to Lembata. This suggests the possibility that the dispersal from West Flores to Lembata may have occurred over the temporary land bridges that, according to current channel depths, would have fused Flores and Lembata. However, given the pervasive tectonic and volcanic activity along with high levels of erosion, the current channel depths are not likely to be reliable guides to historical island separation unless the channels are very large (R. Hall, pers. comm.). The channels separating Flores from

Adonara and Adonara from Lembata are both very shallow and narrow. While both models 2 and 3 show some support for a back-colonization of West Flores (driven by the single West Flores sample sister to Lembata), we find this scenario less likely than a single dispersal event from West Flores to Lembata.

While our best-fitting biogeographical models involve long-distance leap-frog dispersal events to account for the phylogenetic relationships of the major lineages within *L. kadarsani*, this interpretation assumes that Flores Island arose more or less in its present configuration and has remained that way. The formation of the Sunda Arc initially would have consisted of individual emergent volcanoes that would have coalesced into progressively larger and more elongate islands over time (R. Hall, pers. comm.). Though not conclusive, our results are consistent with a scenario in which East Flores arose first, with West Flores emerging at a later time. One such geological boundary proposed in the central region of Flores (just west of Ende) is a zone of decoupling due to the differential motions of the Sunda Arc and southern Banda Arc (Fortuin, Van der Werff, & Wensink, 1997; Katili, 1975; Shulgin et al., 2009). This decoupling boundary, along with the extinct volcanic activity in West Flores (Hall & Spakman, 2015), suggests the possibility that this could be the site where East and West Flores coalesced. Such a history could explain our best-fitting model, which requires an initial long-distance dispersal event from Sumbawa to East Flores, which seems more plausible if there was no intervening land present at that time. Similarly, if West Flores later arose as a separate island closer to Sumbawa than to East Flores, this would be consistent with an independent invasion of West Flores from Sumbawa as per our best-fitting model. This model still requires a leap-frog event from West Flores to Lembata. However, it is possible that the West Flores clade actually extends into East Flores but was missed during our sampling efforts, as both clades appear to overlap in central Flores. This hypothesis will be testable with additional sampling of *L. kadarsani* on Flores, and with the development of more refined models of the geological history of the Inner Banda Arc.

5 | CONCLUSIONS

The mitochondrial phylogenies rendered *L. kadarsani* paraphyletic by placing the Lombok population of *L. kadarsani* as sister to *L. dammermani*, to the exclusion of all other *kadarsani* populations. The pectinate topology of the Bayesian mtDNA tree is consistent with the stepping-stone model of island colonization. However, the nuclear dataset recovered each species as monophyletic sister lineages, with the timing of splits suggesting an ancient mitochondrial introgression event from *L. dammermani* into the Lombok population of *L. kadarsani*, as well as a more recent mitochondrial introgression event from Lembata into East Flores. Our results do not rule out an in situ divergence event on Lombok, rather it suggests that dispersal to the islands east of Lombok came from the *L. kadarsani* lineage. The most supported biogeographical model depicts a colonization sequence of *L. kadarsani* that contains multiple leap-frog dispersals and suggests that Flores has been colonized from Sumbawa twice. While biogeographical studies

of other taxa are needed to better understand the common factors affecting the divergence of populations among the Lesser Sundas, this study has shown a possible example of in situ speciation, multiple long-distance leap-frog dispersal events, multiple signatures of mito-chondrial introgression that by chance created a stepping-stone pattern in the mtDNA phylogeny and parapatrically distributed non-sister lineages occurring on Flores. We show conclusively that the currently accepted stepping-stone model of Lesser Sundas biogeography is insufficient to explain the diversification histories of the entire fauna. Given our findings, we propose that each of the major lineages of *Limnonectes* occurring on the islands east of Lombok be elevated to species status given their genetic distinctiveness and lack of connectivity with other *Limnonectes* in the archipelago. The species diversity of terrestrial vertebrates in this region is almost certainly underestimated, and comprehensive phylogenomic studies of other Lesser Sundas taxa will likely uncover additional undescribed species.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ACCESSIBILITY

Sequence data for the *16S* mitochondrial gene have been submitted to the GenBank database under accession numbers MK079952–MK080107. Sequencing data from the exon-capture experiment have been submitted to the NCBI sequence read archive (SRA) under accession number PRJNA497907. Software input files available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.nk1mt6b.

Title: Data from: Leap-frog dispersal and mitochondrial introgression: phylogenomics and biogeography of Limnonectes fanged frogs in the Lesser Sundas Archipelago of Wallacea

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BIOSKETCH

S.B.R. is interested in the biogeography and conservation of reptiles and amphibians.

SUPPORTING INFORMATION

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