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## Phylogenetic relationships of southern Wallacean ranid frogs (Anura: Ranidae: *Hylarana*)

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### Abstract

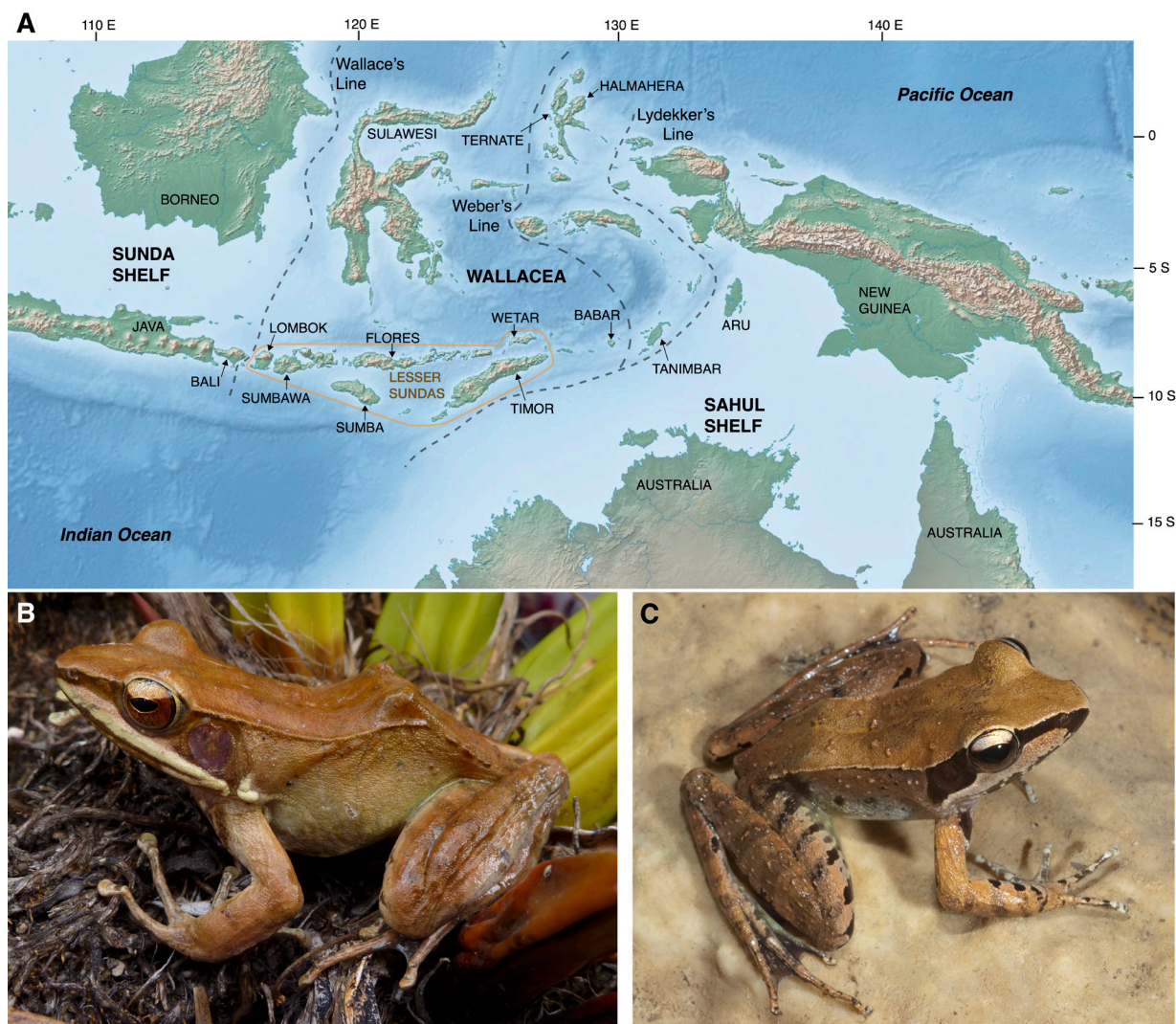
Frogs in the family Ranidae are diverse in Asia and are thought to have dispersed to the Sahul Shelf approximately 10 million years ago, where they radiated into more than a dozen species. Ranid species in the intervening oceanic islands of Wallacea, such as *Hylarana florensis* and *H. elberti* from the Lesser Sundas and *H. moluccana* from eastern Wallacea, are assumed to belong to the subgenus *Papurana*, yet this has not been confirmed with molecular data. We analyzed mitochondrial DNA of *Hylarana* species from five islands spanning the reported ranges of *H. florensis* and *H. elberti* and compared them to confirmed *Papurana* species and closely related subgenera within *Hylarana*. We find that the Lesser Sunda *H. florensis* and *H. elberti* form a clade that is sister to the rest of the Australo-Papuan *Papurana* assemblage. Species delimitation analyses and divergence time estimates suggest that populations of *H. florensis* on Lombok may be distinct from those on Flores at the species level. Likewise, populations of *H. elberti* on Sumba and Timor may be distinct from each other and from those on Wetar, the type locality of *H. elberti*. Samples from Babar Island thought to be members of *H. elberti* in fact belong to the wide-ranging *H. daemeli*, which occurs in northern Australia, across New Guinea, and on the neighboring island of Tanimbar. These results suggest that the Lesser Sundas may have served as a stepping-stone for colonization of the Sahul Shelf and that species diversity of *Papurana* frogs is underestimated in the Lesser Sundas.

**Key words:** Amphibians, Biogeography, Lesser Sundas, Moluccas, Phylogeography

### Introduction

The Sunda and Sahul Shelves are extensions of the Asian and Australian continental shelves, respectively, and they are separated by an archipelago of hundreds of oceanic islands, which form the biogeographic realm known as Wallacea (Fig. 1). Since the initial Wallacean islands emerged ~25 million years ago, they have acted as stepping-stones for flora and fauna to expand into Wallacea from either shelf and sometimes crossing entirely from one continental region to the other (Mayr 1944; Whittaker & Fernandez-Palacios 2007; Lohman *et al.* 2011). The southern portion of Wallacea comprises the Lesser Sunda Archipelago, which is composed of several geologically distinct features. These include the eastern portion of the Sunda Volcanic Arc (e.g., Lombok, Sumbawa, and Flores), an enigmatic continental plate fragment (Sumba), and portions of the Inner (e.g., Wetar) and Outer Banda Arc (e.g., Timor, Babar, and Tanimbar) (Hall 2009, 2011). The Lesser Sundas are closely spaced compared to other major islands in Wallacea, which has promoted intra-archipelago dispersal while acting as a two-way filter between the continental shelf biotas (Darlington 1957; Whittaker & Fernandez-Palacios 2007). The islands that emerged first in the Lesser

Sundas are in the volcanic Sunda Arc portion of the archipelago (Lombok, Sumbawa, Flores) and are estimated to have arisen as early as 10–12 million years ago (My), while the major non-volcanic islands of Sumba and Timor are estimated to be younger at around 3–4 My (Fortuin *et al.* 1997; Haig & McCartain 2007; Nguyen *et al.* 2013; Tate *et al.* 2014).



**FIGURE 1.** A) Map of the Wallacea region bound to the west by Wallace's Line and to the east by Lydekker's Line with the Lesser Sunda archipelago outlined in light orange. Weber's line represents the faunal balance line between islands with  $\geq 50\%$  Asian origin species and islands with  $\geq 50\%$  Australo-Papuan origin species. B) *Hylarana florensensis* from Lombok island (Photo: S. Reilly). C) *Hylarana elberti* from Sumba Island (Photo: J. McGuire).

Though frogs are generally intolerant of salt water at concentrations found in the ocean, a number of anuran species have colonized the oceanic islands of Wallacea (which are separated from the continental shelves by deep-water straits), with some even crossing it entirely reaching the opposing continental shelf. One anuran clade that ranges across Southeast Asia (Sunda Shelf), Wallacea, and Australo-Papua (Sahul Shelf) is an assemblage of ranid frogs in the widespread genus *Hylarana* (a clade that extends from Africa, across Asia, and into Australasia). *Hylarana* included 11 subgenera prior to a molecular study by Oliver *et al.* (2015), most of which were temporarily elevated to generic status and some of which were synonymized. Based on their phylogenetic estimate, Oliver *et al.* (2015) placed all former *Hylarana* species from New Guinea, Australia, and the Solomon Islands in the genus *Papurana*. Notably, their study did not include samples from Wallacea, which is inhabited by several currently recognized species, including the former *Hylarana florensensis* (Lombok, Sumbawa, Flores), *H. elberti* (Sumba, Timor, Wetar, and implicated to occur on Babar and Tanimbar; Menzies 1987, Oliver *et al.* 2015), and *H. moluccana* (Ternate, Halmahera; Oliver *et al.* 2015, Frost 2021). Because Dubois (1992) had placed these three species into *Papurana*, Oliver *et al.* (2015) also provisionally placed them in *Papurana*—with the caveat that this designation required



further testing with molecular and morphological data. A phylogenomic analysis of the group by Chan *et al.* (2020) further expanded the genus by including *Indosylvirana milleti*, *Hylarana attigua*, and *H. celebensis* in the genus *Papurana*, but these authors also did not include species from the Lesser Sundas or Maluku. Most recently, Dubois *et al.* (2021) placed all of the elevated genera (including *Papurana*, but excluding *Abavorana*) back into *Hylarana* relegating those ranks as subgenera. Thus, the current conception of the subgenus *Papurana* includes ranid frogs that extend from the Sunda Shelf, across Wallacea, and into Australia, New Guinea, and the Solomon Islands, with the Lesser Sundas and Maluku species included provisionally.

In this study, we sequence the *16S* mitochondrial gene included in the data set of Oliver *et al.* (2015) to infer the phylogenetic placement of southern Wallacean ranid frogs, including *Hylarana florensis*, *H. elberti*, and *H. cf. elberti* from Babar Island, as well as a population of unidentified ranid frogs from Aru Island, which is a part of the Sahul Shelf. Morphological examination was conducted on these specimens to determine if they fit the diagnostic morphological description of the subgenus *Papurana* (sensu Oliver *et al.* 2015), and of the species they are assigned to, though identification to species based purely on morphological characters can be notoriously difficult in this group (Kraus and Allison 2007). We utilize a Maximum Likelihood phylogenetic estimate paired with single-locus species delimitation analysis to infer the relationships between these populations and species, and to generate hypotheses about the species-level status of individual island populations. We then employ a time-calibrated Bayesian phylogeny to provide insight into the biogeographic patterns within the group and delineate the approximate timing of colonization events.

## Materials and methods

Field expeditions were undertaken to Lombok, Flores, Sumba, Timor, Babar, and Aru for sample collection of ranid frog specimens. Initial identification of species was premised on published literature records for each island and preliminary morphological assessment of specimens. Morphological diagnosis of the subgenus *Papurana* according to Oliver *et al.* (2015) includes the following characters: 1) having a postocular eye mask, 2) robust body shape, 3) strong vermiculations on the posterior surface of the thighs, and 4) dorsolateral folds either absent or thin, with asperities. Examination of these characters was undertaken on the newly collected specimens to determine if they fit the description of the subgenus *Papurana*.

Preserved specimens and tissue samples are housed at either the Museum of Vertebrate Zoology, University of California, Berkeley (MVZ), or the United States National Museum of Natural History, Smithsonian Institution (USNM). The Qiagen DNeasy blood and tissue kit was used to extract genomic DNA from liver tissue (Qiagen, Valencia, California, USA). A portion of the *16S* ribosomal RNA gene was PCR-amplified for samples of *Hylarana florensis* and *H. elberti* using the primers 16sc-L (5'-GTRGGCCTAAAAGCAGCCAC-3') and 16sd-H (5'-CTCCGGTCTGAACTCAGATGACGTAG-3') and for Aru and Babar samples using 16sc-L and 16sb-H (5'-CCGGTCTGAACTCAGATCACGT-3') to amplify a shorter fragment because we were unable to amplify the entire gene region using the original primer pair (Palumbi *et al.* 1991). PCR products were cleaned with ExoSAP-IT (USB, Cleveland, Ohio, USA) and then used in a cycle sequencing reaction using BigDye v3.1 (Applied Biosystems, Foster City, California, USA) for the forward and reverse primers. Cycle sequencing products were cleaned using ethanol precipitation and sequenced on an ABI 3730 automated sequencer (Applied Biosystems). Forward and reverse sequence reads were combined in GENEIOUS v11.1.5 (www.geneious.com). *16S* sequence data from the subgenera *Papurana*, *Hydrophylax*, *Indosylvirana*, and *Sylvirana* used by Oliver *et al.* (2015) were downloaded from GenBank and a fragment of *16S* assembled from a next-generation sequencing experiment was included from *H. celebensis* (GenBank SRA BioProject PRJNA631853; Chan *et al.* 2020). These sequences were aligned with MUSCLE (Edgar 2004), and the final alignment length was 808 bp. All newly generated *16S* sequences have been deposited in GenBank (accession numbers OL960055-OL960074).

Maximum Likelihood (ML) phylogenetic analysis was carried out using IQTREE v1.6.12 (Nguyen *et al.* 2015). The best fit model of sequence evolution according to Bayesian information criterion (BIC) scores was TIM2+F+I+G4 (Kalyaanamoorthy *et al.* 2017) and node support was assessed with 1000 ultrafast bootstrap replicates (UFBoot; Hoang *et al.* 2017) and 1000 single branch test (SH-aLRT) replicates. Species delimitation hypotheses were estimated using a method that builds and ranks species partitions from single locus sequence alignments (ASAP; Puillandre *et al.* 2021). Divergence times were estimated using the Bayesian multispecies coalescent program STARBEAST2

(Ogilvie *et al.* 2017) with a strict molecular clock and a rate of 1.3% divergence/million years which was estimated for the *16S* gene in North American ranid frogs (Macey *et al.* 2001). Two runs of 50 million generations, sampled every 5000 generations, were carried out and log files were viewed in TRACER v1.7 (Rambaut *et al.* 2018) to assess parameter convergence by confirming that effective sample size (ESS) values are > 200. After discarding 10% of the samples as burn-in, the remaining trees were combined to create a maximum clade credibility tree with node support assessed as posterior probability values (PP). The uncorrected sequence divergence between relevant lineages was estimated with the software DIVEIN (Deng *et al.* 2010).

**TABLE 1.** Sample locality and GenBank information for newly sequenced or assembled samples in this study.

Species	Catalog #	Collector #	Island	Lat	Long	GenBank ID
<i>H. florensis</i>	MVZ 292867	JAM 11947	Lombok	-8.4027	116.5396	OL960057
<i>H. florensis</i>	MVZ 292868	JAM 12000	Lombok	-8.3078	116.4048	OL960056
<i>H. florensis</i>	MVZ 292869	JAM 12001	Lombok	-8.3078	116.4048	OL960058
<i>H. florensis</i>	MVZ 292865	JAM 12523	Flores	-8.7595	121.7009	OL960055
<i>H. elberti</i>	MVZ 292834	JAM 13083	Sumba	-10.0210	120.0580	OL960060
<i>H. elberti</i>	MVZ 292835	JAM 13084	Sumba	-10.0210	120.0580	OL960062
<i>H. elberti</i>	MVZ 292836	JAM 13085	Sumba	-10.0210	120.0580	OL960061
<i>H. elberti</i>	MVZ 292837	JAM 13086	Sumba	-10.0210	120.0580	OL960059
<i>H. elberti</i>	MVZ 292838	JAM 13087	Sumba	-10.0210	120.0580	OL960063
<i>H. elberti</i>	USNM 579403	-	Timor	-8.7833	125.4500	OL960067
<i>H. elberti</i>	USNM 579404	-	Timor	-8.7833	125.4500	OL960068
<i>H. elberti</i>	USNM 579405	-	Timor	-8.7833	125.4500	OL960065
<i>H. elberti</i>	USNM 579406	-	Timor	-8.7833	125.4500	OL960064
<i>H. elberti</i>	USNM 579407	-	Timor	-8.7833	125.4500	OL960066
<i>H. daemeli</i>	MVZ 295042	ALS 825	Babar	-7.8780	129.6168	OL960069
<i>H. daemeli</i>	MVZ 295043	ALS 826	Babar	-7.8780	129.6168	OL960070
<i>H. daemeli</i>	MVZ 295044	ALS 827	Babar	-7.8780	129.6168	OL960071
<i>H. daemeli</i>	MVZ 295045	ALS 837	Babar	-7.8780	129.6168	OL960072
<i>H. daemeli</i>	MVZ 295046	ALS 838	Babar	-7.8780	129.6168	OL960073
<i>H. arfaki</i>	MVZ 273849	ALS 305	Aru	-6.5028	134.3914	OL960074

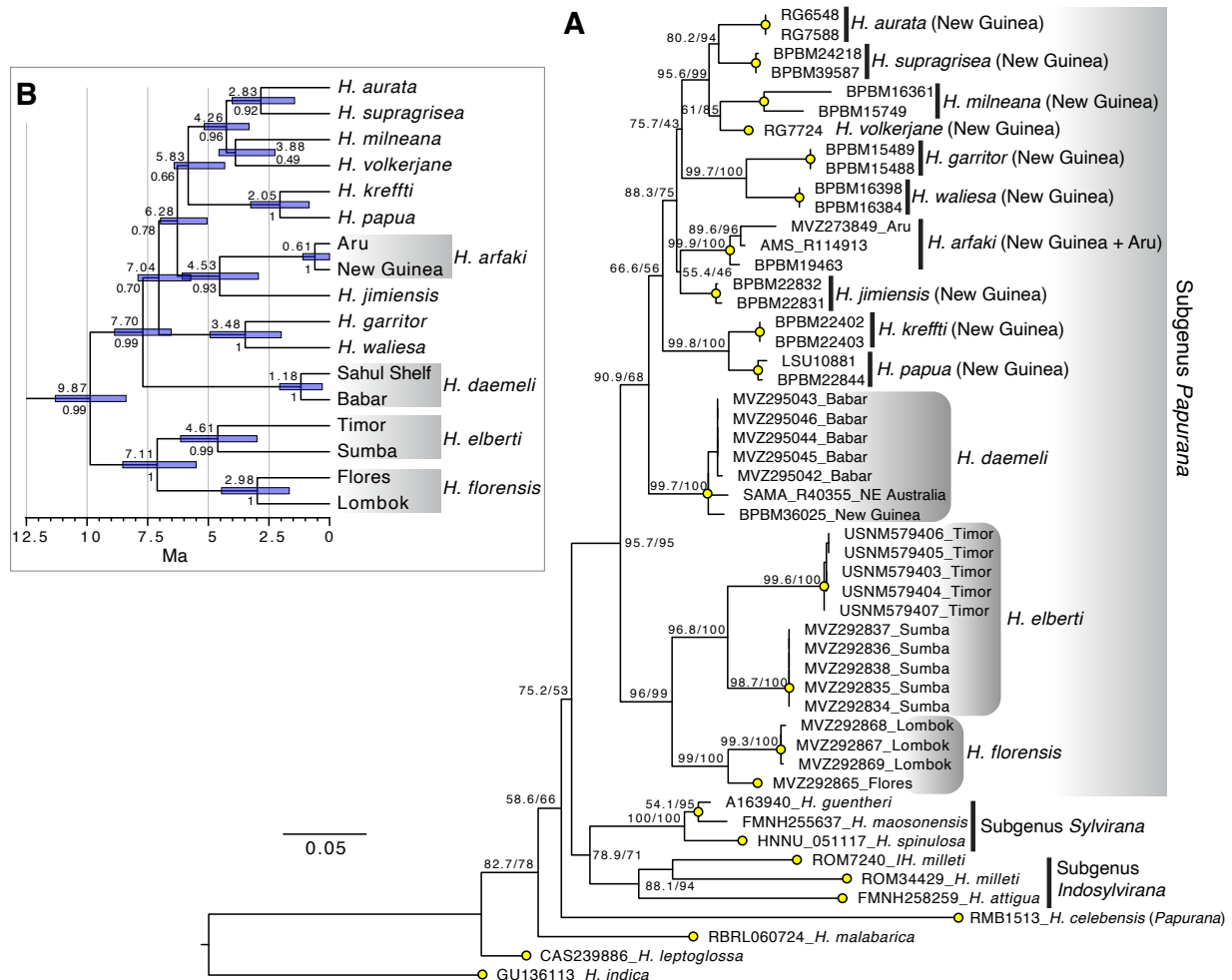
## Results

Our visual examination of specimens from the ranges of *H. florensis* and *elberti* are consistent with the diagnostic characters for the subgenus *Papurana* as described in Oliver *et al.* (2015). We note that the posterior thighs did not always contain strong vermiculations as stated in the diagnosis. However, in table 3 of Oliver *et al.* (2015) they state that the posterior thighs of the subgenus *Papurana* are “vermiculated to finely mottled, but variable among species.” The posterior thighs of the *H. florensis* specimen from Flores were very finely mottled or almost not mottled at all, though it is unclear if this is due to preservation and/or storage conditions. While the bars on the dorsal thigh of the Flores specimen were absent in comparison to specimens from Lombok, Oliver *et al.* (2015) state that thigh bars in the subgenus *Papurana* can either be present or absent. Examination of the Babar specimens is consistent with the description of the subgenus *Papurana* as well, and within the limits of intraspecific variation for *H. daemeli*.

The uncorrected average *16S* sequence divergence between the Lesser Sundas group and the Australo-Papuan group is 10.5%, and between *H. florensis* and *H. elberti* is 9.0%. The uncorrected average sequence divergence between individual southern Wallacean island populations can be found in Table 2, with particularly relevant values including 4.2% divergence between Lombok and Flores, 7.0% divergence between Sumba and Timor, and 1.2% divergence between Babar and Sahul Shelf *H. daemeli*.

Phylogenetic analyses (Fig. 2) identify the Lombok and Flores populations of *H. florensis* as sister lineages (UFBoot = 99, SH-aLRT = 100, PP = 1.00) and the Sumba and Timor populations of *H. elberti* as sister lineages

(UFBoot = 96.8, SH-aLRT = 100, PP = 0.99). *Hylarana florensis* is recovered with strong support as sister to *H. elberti* (UFBoot = 96, SH-aLRT = 99, PP = 1), forming a Lesser Sunda clade that is sister to the rest of the subgenus *Papurana* as defined by Oliver *et al.* (2015). The Babar samples that were assumed to be *H. elberti* are recovered as nested within *H. daemeli* with strong support (UFBoot = 99.7, SH-aLRT = 100, PP = 1.00). The sample from Aru is confirmed as *H. arfaki* (UFBoot = 99.9, SH-aLRT = 100, PP = 1.00). The highest supported species partition contained 25 species and finds each island in the Lesser Sundas as a candidate species, while including the Babar samples in *H. daemeli* (Fig. 2A). Of the top ten species delimitation partitions, all find the Sumba and Timor populations of *H. elberti* to be distinct and 7/10 partitions find the Lombok and Flores populations to be distinct from each other.



**FIGURE 2.** (A) Maximum Likelihood phylogeny of the *16S* mitochondrial gene from IQTREE. Numbers above nodes represent ultrafast bootstrap (UFBoot) values before the slash and single branch tests (SH-aLRT) after the slash where UFBoot  $\geq 95$  and SH-aLRT  $\geq 80$  is considered strong support. Yellow circles at nodes/tips of the tree represent species delimitation hypotheses from the software ASAP. (B) Time-calibrated STARBEAST2 species tree where numbers above nodes represent the mean divergence time in millions of years ago (Ma) and the numbers below nodes represent posterior probability (PP) support where strong support is considered PP  $\geq 0.95$ . Blue bars at nodes represent 95% confidence intervals for node age. Outgroups are not shown for better visualization of focal clade.

The estimated divergence times (Fig. 2B), which should be cautiously interpreted, estimate the Lesser Sunda clade to have diverged from an Australo-Papuan clade  $\sim 9.9$  million years ago (95% posterior density confidence intervals (CI) 8.4–11.3 Ma). The estimated divergence between *H. florensis* and *H. elberti* is  $\sim 7.1$  Ma (95% CI 8.4–11.3 Ma), with the Lombok and Flores populations of *H. florensis* diverging  $\sim 3$  Ma (95% CI 1.7–4.5 Ma) and the Sumba and Timor populations of *H. elberti* diverging  $\sim 4.6$  Ma (95% CI 3.0–6.1 Ma). The Babar population of *H. daemeli* is estimated to have diverged from the Sahul Shelf populations  $\sim 1.2$  Ma (95% CI 0.3–2.1 Ma), and the Aru population of *H. arfaki* diverged from the included New Guinea populations  $\sim 0.6$  Ma (95% CI 0.1–1.1 Ma).

**TABLE 2.** Average uncorrected *I6S* sequence divergence between individual southern Wallacean island populations.

	<i>H. florensis</i> —Flores	<i>H. elberti</i> —Sumba	<i>H. elberti</i> —Timor	<i>H. daemeli</i> —Babar	<i>H. daemeli</i> —Sahul
<i>H. florensis</i> —Lombok	0.042	0.083	0.098	0.084	0.103
<i>H. florensis</i> —Flores		0.080	0.095	0.073	0.093
<i>H. elberti</i> —Sumba			0.070	0.075	0.096
<i>H. elberti</i> —Timor				0.097	0.111
<i>H. daemeli</i> —Babar					0.012

## Discussion

Until now, the lack of molecular data for the ranid species of southern Wallacea required that taxonomists make an educated guess as to their phylogenetic relationships relative to species from the subgenus *Papurana* in New Guinea, Australia, and the Solomon Islands. We here confirm that the phylogenetic placement and morphology of *Hylarana florensis* and *H. elberti* is consistent with their inclusion in the subgenus *Papurana* (sensu Oliver *et al.* 2015). We show furthermore that *H. florensis* and *H. elberti* in the Lesser Sundas are monophyletic and sister to the Australo-Papuan *Hylarana* (*Papurana*) radiation composed of at least 13 species (AmphibiaWeb 2021). The population from Sumba, which had been treated originally as *H. florensis* (Van Kampen and Brongersma 1931), and not definitively assigned to either *H. florensis* or *H. elberti* since (except for a natural history note; Reilly *et al.* 2016), is genetically confirmed to be most closely related to *H. elberti*. The population from Babar, assumed to belong to *H. elberti* (Menzies 1987), is instead both genetically and morphologically confirmed to be an island population of the wide-ranging *H. daemeli*, which occurs on New Guinea and some of its neighboring islands, northern Australia, and neighboring Tanimbar Island approximately 135 km to the east (specimen WAM-R112256; confirmed as *H. daemeli* by P. Doughty, curator of herpetology at the Western Australian Museum). The type locality of *H. moluccana* is on Ternate Island off the west coast of Halmahera (Boettger 1895), and genetic data from Ternate and Halmahera samples is needed both to confirm its assumed phylogenetic placement within the subgenus *Papurana* and to resolve its lineage status relative to other *Hylarana* species.

Our results also suggest that *H. florensis* and *H. elberti* may each be composed of multiple distinct species that diverged from each other in the late Pliocene. While we lack samples of *H. florensis* from Sumbawa, the Lombok population appears to be a candidate species distinct from Flores, which is the type locality of *H. florensis* (Boulenger 1897). Similarly, the Sumba and Timor populations of *H. elberti* appear to be candidate species, but until they are compared to the population on Wetar, the type locality for *H. elberti* (Roux 1911; Mertens 1967), it is unclear whether either population would retain the name *elberti* in the event of a taxonomic revision. Kaiser *et al.* (2011) reported morphological differences between the Timor population and the holotype of *H. elberti*, suggesting that these populations may represent separate species. Further morphological and multilocus genetic study of these lineages will be needed to inform any taxonomic revision of *H. florensis* and *H. elberti*.

The relationships of species in the base of our phylogeny, those outside of the Lesser Sunda + Australo-Papuan radiation clade, are not well supported and disagrees with Chan *et al.* (2020). The Chan *et al.* (2020) paper used genome-wide data (thousands of loci) compared to our single mitochondrial gene, and as such the Chan *et al.* (2020) topology both within the Australo-Papuan clade and outside of the Lesser Sunda + Australo-Papuan clade should be considered the most well-supported at the current time.

The prior finding that the subgenus *Papurana* originated on the Sunda Shelf (Chan *et al.* 2020) together with our discovery that the Lesser Sundas clade is sister to the Australo-Papuan clade is consistent with a biogeographical scenario, in which the Lesser Sundas served as a stepping-stone between the Asian and Australo-Papuan biogeographic realms. Furthermore, once the Lesser Sundas were colonized, *Hylarana* (*Papurana*) experienced limited insular radiation within it. The first species to branch off the Australo-Papuan clade both in this study and in Oliver *et al.* (2015) is *H. daemeli*, a mainland Sahul species that appears to have back-colonized the Banda Arc islands of Tanimbar and Babar, thereby establishing a boundary between the Lesser Sunda and Australo-Papuan clades in the middle of southeastern Wallacea, between Timor and Babar. This back-colonization hypothesis is supported by the fact that the age of the *H. daemeli* lineage (~7 Ma) is much older than the emergent history of the islands of Babar and Tanimbar (~1-3 Ma; Kaneko *et al.* 2007), while the Sahul Shelf has been emergent during that entire



period. This result reinforces the idea that Babar is near the border of the faunal balance line, or Weber's Line (Fig. 1), which separates islands with 50% or more of Asian origin species from islands with 50% or more of Australo-Papuan origin species and represents the boundary between the Asian and Australo-Papuan biogeographic realms (Pelseneer 1904; Mayr 1944).

Recent molecular studies of southern Wallacean herpetofauna have revealed a wide range of divergence levels and a multitude of biogeographic patterns ranging from recent colonization and expansion through the region without insular species formation (Reilly *et al.* 2017, 2019b; Karin *et al.* 2020; Maryanto *et al.* 2021) to much earlier colonizations that led to extensive species diversification within and between islands (Blom *et al.* 2019; Reilly *et al.* 2019a, 2021, 2022). Among early colonizing taxa, a pattern of disconnect between Sunda Arc island populations and populations on Sumba plus the Banda Arc islands has emerged, suggesting that while Sumba is currently closest to Sumbawa and Flores it may have been in a different position in the past (Reilly 2016; Reilly *et al.* 2022). Our finding that Sumba *H. elberti* are more closely related to Timor than to *H. florensis* group populations adds to evidence supporting this biogeographic pattern. An additional repeated pattern that is emerging is that Timor and Sumba have very old lineage ages for populations. We estimate that the Sumba and Timor populations diverged ~3-6 Ma, which is comparable to the estimated emergent ages of the islands at approximately 3-4 Ma (Fortuin *et al.* 1997; Haig & McCartain 2007; Nguyen *et al.* 2013; Tate *et al.* 2014). Continued research into the timing of island colonization by other taxa in the Lesser Sundas will improve our understanding of the biogeographical and geological history of the archipelago.

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