

# Adaptive Radiation and Ecological Opportunity in Sulawesi and Philippine Fanged Frog (*Limnonectes*) Communities

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**ABSTRACT:** Because island communities are derived from mainland communities, they are often less diverse by comparison. However, reduced complexity of island communities can also present ecological opportunities. For example, amphibian diversity on Sulawesi Island is lower than it is in the Philippines, but Sulawesi supports a surprising diversity of Sulawesi fanged frogs (*Limnonectes*). Here we examine molecular, morphological, and geographical variation of fanged frogs from these two regions. Using genealogical concordance, morphology, and a Bayesian approach to species delimitation, we identified 13 species on Sulawesi, only four of which have been previously described. After evolutionary history is accounted for, a model with multiple body size optima in sympatric species is favored over a “random-walk” model of body size evolution. Additionally, morphological variation is higher among sympatric than nonsympatric species on Sulawesi but not in the Philippines. These findings suggest that adaptive radiation of fanged frogs on Sulawesi was driven by natural selection to infiltrate ecological niches occupied by other frog lineages in the Philippines. This supports a role of ecological opportunity in community assembly: diversification in mature communities, such as the Philippines, is limited by a dearth of unoccupied ecological niches. On Sulawesi, evolutionary novelties originated in a predictable and replicated fashion in response to opportunities presented by a depauperate ancestral community.

## Introduction

An adaptive radiation is the rapid evolution of ecological or phenotypic diversity within a closely related group of organisms (Schluter 2000b; Kassen 2009; Glor 2010; Losos 2010; Losos and Mahler 2010). Adaptive radiations are evinced by (1) a closely related (but not necessarily mono-

phyletic) suite of species that (2) rapidly evolved and (3) are characterized by diverse and functionally useful phenotypes that (4) are correlated with their environment (Schluter 2000b). Recent studies have concluded that various groups have adaptively radiated, resulting in novel ecological communities structured on key resources (Schluter 2000b). For example, variation in beak morphology among species allowed Darwin’s finches to access different food resources in the Galapagos Islands (Grant 1999), variation in social systems and life history of cichlid fish species catalyzed occupancy of a diverse array of microhabitats in Lake Victoria (Martens 1997; Verheyen et al. 2003), and repeated evolution of body morphology in lizards of the genus *Anolis* contributed to their diversification in microhabitats of the Greater Antilles (Losos et al. 1998). Adaptive radiations may be characterized by periods of particularly rapid speciation (a temporal burst), which could result in an adaptively radiated clade with extraordinarily high species diversity relative to another similarly aged group (Glor 2010). However, this is not necessarily the case: for example, adaptive radiation in the Galapagos Islands yielded only ~14 species of Darwin’s finches (Grant and Grant 2008), which represents a level of diversity that is not particularly extraordinary. More prominently, adaptive radiations are accompanied by pronounced and ecologically relevant phenotypic diversification. Such phenotypic diversification can create or be a consequence of ecological opportunity, a surplus of accessible resources that are unexploited by competing taxa (Simpson 1953; Schluter 2000b; Losos 2010). Ecological opportunity is associated with relaxed stabilizing selection, and the evolutionary response to this relaxed selection is known as ecological release (Losos and de Queiroz 1997; Yoder et al. 2010).

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In this study, we investigated another putative example of an adaptive radiation involving fanged frogs (genus *Limnonectes*) on the Indonesian island of Sulawesi. *Limnonectes* species occur in South and Southeast Asia and have been the subject of morphological (Emerson and Voris 1992; Emerson 1994, 1996; Emerson and Ward 1998) and phylogenetic (Emerson and Berrigan 1993; Emerson et al. 2000; Evans et al. 2003c) studies motivated by their interesting reproductive ecology, broad distribution, high diversity, and cryptic morphological variation among species. This genus includes species with reversed sexual dimorphism (larger males; unusual in frogs); viviparity (Iskandar and Tjan 1996); nest attendance (Brown and Iskandar 2000); male territoriality, fangs, and combat (Emerson 1992; Orlov 1997; Tsuji and Lue 1998); and purported male voicelessness (Emerson 1992).

Sulawesi is an island in central Indonesia with an area ~60% of the size of the Philippine Archipelago (fig. 1), so species diversity is expected by island biogeographical theory to be lower on Sulawesi (MacArthur and Wilson 1967). This prediction is generally borne out in frogs at the family, genus, and species levels (Iskandar and Tjan 1996; Alcalá and Brown 1999). Additionally, for frogs, the marine barrier between Sulawesi and Borneo appears to have been more difficult to cross than the marine barriers between Borneo and Palawan, between Palawan and the oceanic islands of the Philippines, and between Borneo and Mindanao via the Sulu Archipelago (Esselstyn et al. 2010). As a consequence, there are no frog genera with representatives on Sulawesi that are not also found in the Philippines, whereas there are 13 genera present in the Philippines that are absent from Sulawesi: *Leptobrachiium* and *Megophrys* (Megophryidae), *Barbourula* (Bombinatoridae), *Ansonia* and *Pelophryne* (Bufonidae), *Kalophrynus* and *Chaperina* (Microhylidae), *Platymantis* (Ceratobatrachidae), *Nyctixalus*, *Philautus* (Rhacophoridae), *Ingerana*, *Hoplobatrachus* (Dicroglossidae), and *Staurois* (Ranidae; Inger 1999; Brown 2007).

An exception to this general pattern of higher frog diversity in the Philippines compared with on Sulawesi occurs in the fanged frog genus *Limnonectes*: ~13 endemic *Limnonectes* species are found in the Philippines, and ~13 different endemic species (one whose distribution extends to the Moluccas) are found on Sulawesi (Iskandar and Tjan 1996; Inger 1999; Evans et al. 2003c; this study). In the Philippines, *Limnonectes* species are a major component of the riparian amphibian fauna but only a minor component of the nonriparian amphibian fauna. On Sulawesi, *Limnonectes* species are prominent elements of both habitats. This suggests that this group may have diversified more quickly or for a longer period on Sulawesi, more slowly or for less time in the Philippines, or some combination of these alternatives. However, phylogenetic anal-

ysis of mitochondrial DNA sequences has indicated that several *Limnonectes* lineages have resided as long or longer in the Philippines than on Sulawesi (Evans et al. 2003c), suggesting that a briefer period for diversification in the Philippines cannot account for a level of species diversity similar to that on Sulawesi. Additionally, fanged frogs dispersed to the Philippines three or four times and to Sulawesi only twice (Evans et al. 2003c), so differences in connectivity between these biogeographical provinces and the adjacent Sunda shelf also cannot explain the similar level of diversity on Sulawesi.

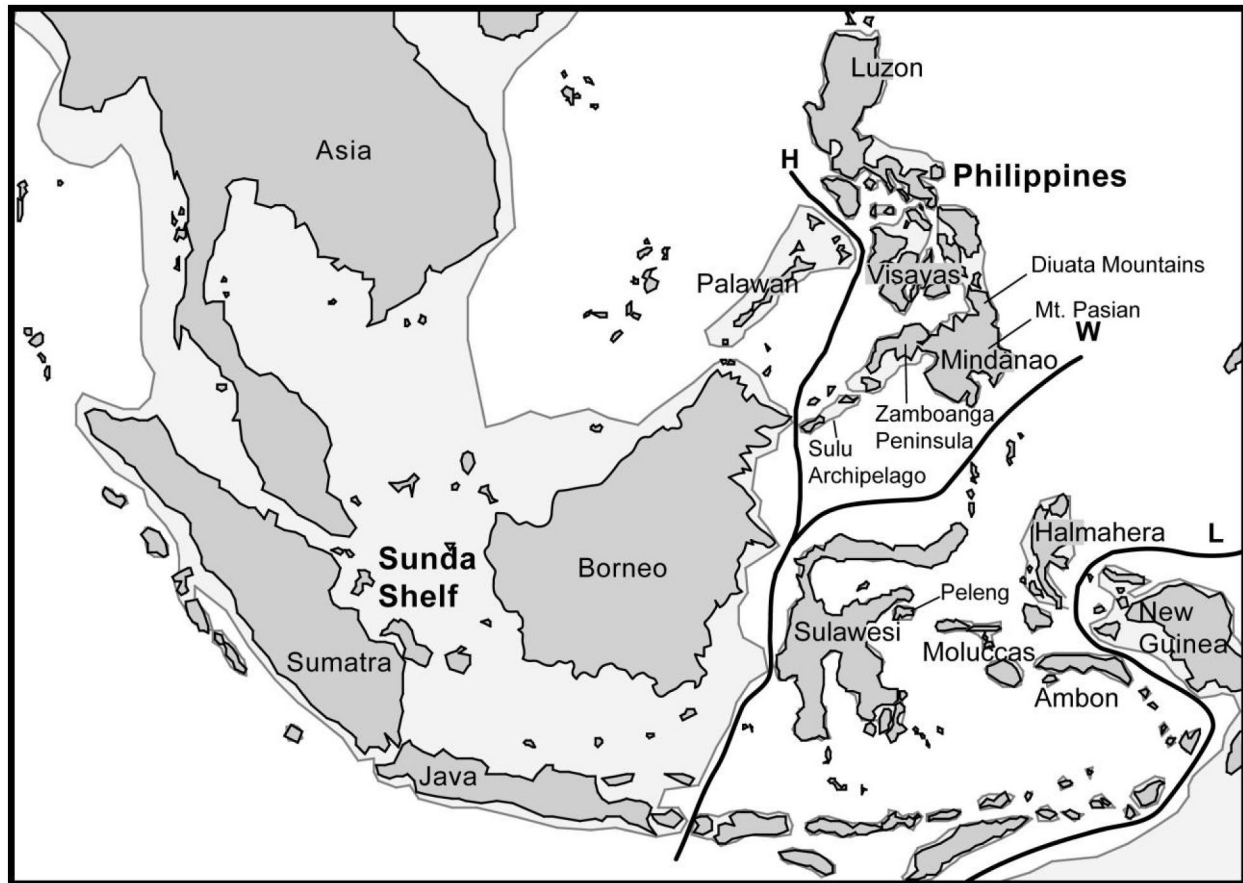
In theory, the paucity of other frog lineages on Sulawesi could have left available ecological niche space (unoccupied habitat, reduced competition, exploitable food resources, etc.), thereby creating ecological opportunity for an adaptive radiation. Our field observations provide anecdotal evidence for niche partitioning in Sulawesi fanged frogs. For example, large-bodied species (e.g., undescribed species *Limnonectes* sp. *D* and *Limnonectes* sp. *I*) are associated with fast-moving water and never occur in sympatry, medium-sized species (e.g., *Limnonectes* sp. *G2*, *Limnonectes modestus*) are associated with more slowly moving water, and small-bodied species (e.g., *Limnonectes arathooni*, *Limnonectes* sp. *I*, *Limnonectes* sp. *V*) are found in leaf litter in forests near rivers. Variation in other morphological and life-history traits (the extent of webbing, variation in reproductive modes) also corresponds to these microhabitats.

In this article, we aim to test the hypothesis that fanged frog diversity on Sulawesi is an example of an adaptive radiation, and to illustrate this point we provide comparisons to the equally diverse *Limnonectes* communities of the Philippines. To this end, our goals here are (a) to comprehensively characterize the diversity of fanged frogs on Sulawesi in terms of the number of putative species and their ranges and (b) to test whether their patterns of biogeographical and morphological diversification are consistent with the hypothesis of an adaptive radiation (Schluter 2000b; Losos and Mahler 2010). More specifically, we tested (1) whether a model with multiple body size optima among sympatric species was preferred over a random-walk model and (2) whether sympatric species on Sulawesi tended to be more morphologically diverged than allopatric species after controlling for the effect of evolutionary relationships.

## Methods

### *Species Concept and Criteria*

We adopt here a general lineage concept of species, wherein a species is defined as a separately evolving lineage of connected subpopulations (metapopulations) through



**Figure 1:** The geographic setting of Sulawesi and the Philippines. Light gray areas indicate the distribution of land and sea during late Pleistocene sea level reductions of 120 m (Voris 2000). Wallace's 1863 (W), Huxley's (H), and Lydekker's (L) biogeographical lines are indicated, as are various islands and mountains referenced in the text. Modified from Evans et al. (2003c).

time (de Queiroz 1998, 2007). As evidence for lineage separation, we use molecular and morphological (body size) criteria to define putative species. We employ a genealogical-concordance criterion in order to identify putative independent lineages. With this approach, lineages (and thus species) are identified by monophyly of multiple unlinked and nonpistatic markers (Avise and Ball 1990; Baum and Shaw 1995; Hudson and Coyne 2002). We base this on estimated genealogies of four mitochondrial genes (analyzed as a single concatenated locus) and one nuclear gene (recombination activating gene 1 [*RAG1*]). Application of the genealogical-concordance criterion provides a conservative estimate of lineage status, because it will fail to identify species that evolved so recently that they exhibit incomplete lineage sorting at one or more loci (Sites and Crandall 1997; Avise 2000). In support of this, we encountered one example described below for which clear morphological differences were observable between

groups that did not fulfill the standard of the genealogical species criterion.

We also used a new Bayesian species delimitation approach (described below; Yang and Rannala 2010) to test species boundaries. While we provide support for multiple new species of fanged frogs on Sulawesi, formal descriptions of these species, including a comprehensive description of morphological characters and ecology, are beyond the scope of this article and are in preparation for publication elsewhere (D. T. Iskandar, unpublished data).

#### *Molecular Data*

We analyzed molecular variation in 697 fanged frogs, including 683 individuals from Sulawesi and 14 individuals from other parts of Southeast Asia (one outgroup sequence from Borneo and ingroup sequences including 10 samples from the Philippines, one from Halmahera, one from Am-

bon, and one from New Guinea), that were selected on the basis of a previous phylogenetic analysis of molecular variation of this genus (Evans et al. 2003c). A Bornean sample of *Limnonectes leporinus* was selected as an outgroup on the basis of this study (Evans et al. 2003c).

Only four endemic species of *Limnonectes* have been described from Sulawesi (*Limnonectes modestus*, *Limnonectes heinrichi*, *Limnonectes arathooni*, and *Limnonectes microtypanum*). *Limnonectes palavanensis* and *Limnonectes dammermanni* have been reported to occur on Sulawesi (Smith 1927; Mertens 1929), but our analyses do not support the continued recognition of these taxa on Sulawesi (D. T. Iskandar, unpublished data). A previous molecular analysis identified additional diverged lineages on Sulawesi (Evans et al. 2003c). As a first step toward providing informal but reliable means to recognize these putative species, Evans et al. (2003c) applied placeholder names to monophyletic groups of individual mtDNA sequences (*Limnonectes* sp. I, *Limnonectes* sp. D, *Limnonectes* sp. T, *Limnonectes* sp. G2, *Limnonectes* sp. V, *Limnonectes* sp. 1, and *Limnonectes* sp. 2). Here we use these informal designations, as well as two new informal names (*Limnonectes* sp. J and *Limnonectes* sp. J2; see below), to refer to putative *Limnonectes* species from Sulawesi. In this study, we included samples from all Sulawesi species and divergent populations reported by Evans et al. (2003c), plus additional samples that were amassed over multiple subsequent expeditions, the localities of which are listed in a supplementary table 1 ("Locality Information") that is available in the Dryad database (<http://dx.doi.org/10.5061/dryad.8913>).<sup>1</sup>

We collected molecular and morphological data from all Sulawesi fanged frog species except *Limnonectes* sp. J and *Limnonectes* sp. J2, from which only molecular data were collected. We collected molecular and morphological data for all Philippine fanged frog species except *Limnonectes diuatus*, *Limnonectes feneri*, and *Limnonectes micrixalus*. *Limnonectes diuatus* is known from the Diuata Mountains of Mindanao, *L. feneri* was recently described and occurs on Mount Pasian on the eastern portion of Mindanao (Siler et al. 2009), and *L. micrixalus* is from Basilan and the tip of the Zamboanga peninsula of western Mindanao (fig. 1) and is known from only a single paratype. Thus, we lack data from a few species with small ranges.

Mitochondrial DNA (mtDNA) sequences were collected from two regions of this genome. For almost all individuals, up to 646 base pairs (bp) were obtained from the cytochrome oxidase I gene (*COI*). Additionally, approxi-

mately ~2,122 bp of 12S and 16S rDNA genes and the intervening tRNA<sup>val</sup> on the mtDNA with unambiguous alignment was sequenced from 50 individuals, including 39 from Sulawesi (Evans et al. 2003c). Thus, the amount of mtDNA sequence collected from each individual ranged from ~600 to 2,700 bp. Approximately 890 bp of nuclear DNA sequences were obtained from the recombination activating gene 1 (*RAG1*) from 158 representative individuals chosen on the basis of inferred relationships in mtDNA, including 145 samples from Sulawesi, 10 from the Philippines, two from the Moluccas, and the Bornean outgroup species *L. leporinus*. Because *RAG1* is an autosomal locus, a few individuals were encountered that were heterozygous for single-nucleotide polymorphisms (SNPs) on diverged alleles. We used one-letter ambiguity symbols to represent both types of nucleotides at a site. Mitochondrial DNA sequences were amplified and sequenced, using published primers (Evans et al. 2003c; Ivanova et al. 2006). New primers were used to amplify *RAG1*: Rana.rag1.rev2.2690 (5' GCA CGA AGA GCG CCA TAC TG 3'), Rana.rag1.for2.2115 (5' ATT TAT CTT TCG TGG TAC AGG 3'), Rana.rag1.for1 (5' TGA TGA ATA CCC AGT GGA GGC TGT TTC TAA AAG G 3'), and Rana.rag1.rev3.2674 (5' TAC TGG TTT CAT TTT GAG GTA 3'). These data have been deposited in Genbank (accession numbers JF743762–JF744624).

#### Phylogenetic Analysis

The mtDNA gene regions were concatenated and divided into partitions (*COI* codons, stem regions of rDNA, loop regions of rDNA) with separate substitution models for phylogenetic analysis. A doublet model was used for the rDNA stem partition, with secondary structure inferred from a model based on the frog *Xenopus laevis* (Cannone et al. 2002). The Akaike Information Criterion (AIC) was used to select models for the loop partition and for each of three codon position partitions for the *COI* locus, using the program MrModel, version 2 (Nylander 2004). For each of these partitions, the general time reversible plus a proportion of invariant sites and a gamma-distributed rate heterogeneity (GTR + I +  $\Gamma$ ) model was selected.

Two partitioning schemes were evaluated for *RAG1*. In the first, the *RAG1* data were analyzed, using the GTR + I +  $\Gamma$  model, which was also selected by the AIC. In the second, a separate GTR + I +  $\Gamma$  model was used for each codon position. These *RAG1* analyses were compared, using Bayes factors as in Nylander et al. (2004). Bayesian analyses of the mtDNA and *RAG1* data sets were performed, using MrBayes, version 3.2.1 (Huelsenbeck and Ronquist 2001), with 10 million generations, and two Markov chain Monte Carlo (MCMC) runs starting with different trees, with Metropolis coupling, using four chains

<sup>1</sup> In the appendix of Evans et al. (2003c), museum catalog AMNH167169 is listed as *Limnonectes* sp. 2 when it should be *Limnonectes* sp. 1; this error is corrected here.

per run, with state swapping among chains controlled by a temperature parameter, which was set at 0.2.

Theory predicts that MCMC runs will eventually converge to a stationary distribution that is equal to the posterior distribution (Brooks and Roberts 1998). Convergence was assessed, using postrun diagnostics, including plots of the tree likelihood, inspection of the average standard deviation of split frequencies, and calculation of the effective sample size, using Tracer, version 1.5 (Drummond and Rambaut 2007). While some challenges were encountered with the mtDNA analysis (discussed below), on the basis of these diagnostics, we discarded as burn-in 5 million generations for the mtDNA analysis and 2 million generations for the *RAG1* analysis.

*Bayesian Species Delimitation.* We used a Bayesian approach for species delimitation as implemented by the program BP&P, version 2.0 (Rannala and Yang 2003; Yang and Rannala 2010). This method estimates posterior probabilities of a species tree, given multilocus molecular data. The approach begins with a user-specified “guide tree” that may be “hyperresolved” with respect to the species tree, for example, by elevating a population-level relationship to a species-level relationship (in other words, by including more tip lineages than there are species represented by the data). Using reversible-jump MCMC, the method explores the set of species trees that can be generated by collapsing nodes in the guide tree. This analysis assumes that population size is constant within each lineage of the species tree, that the guide tree either is the species tree or can be collapsed into the species tree, that each species is panmictic (no population structure), and that gene flow does not occur after speciation. These assumptions are unrealistic if hybridization occurs or if population structure is present, and as a result (especially because of population structure), we view the results of this analysis as a descriptive exercise. Consequently, we relied on the genealogical-concordance criterion—with one exception, where a pair of morphologically distinct species did not meet the genealogical-concordance criterion—as our gold standard for species status.

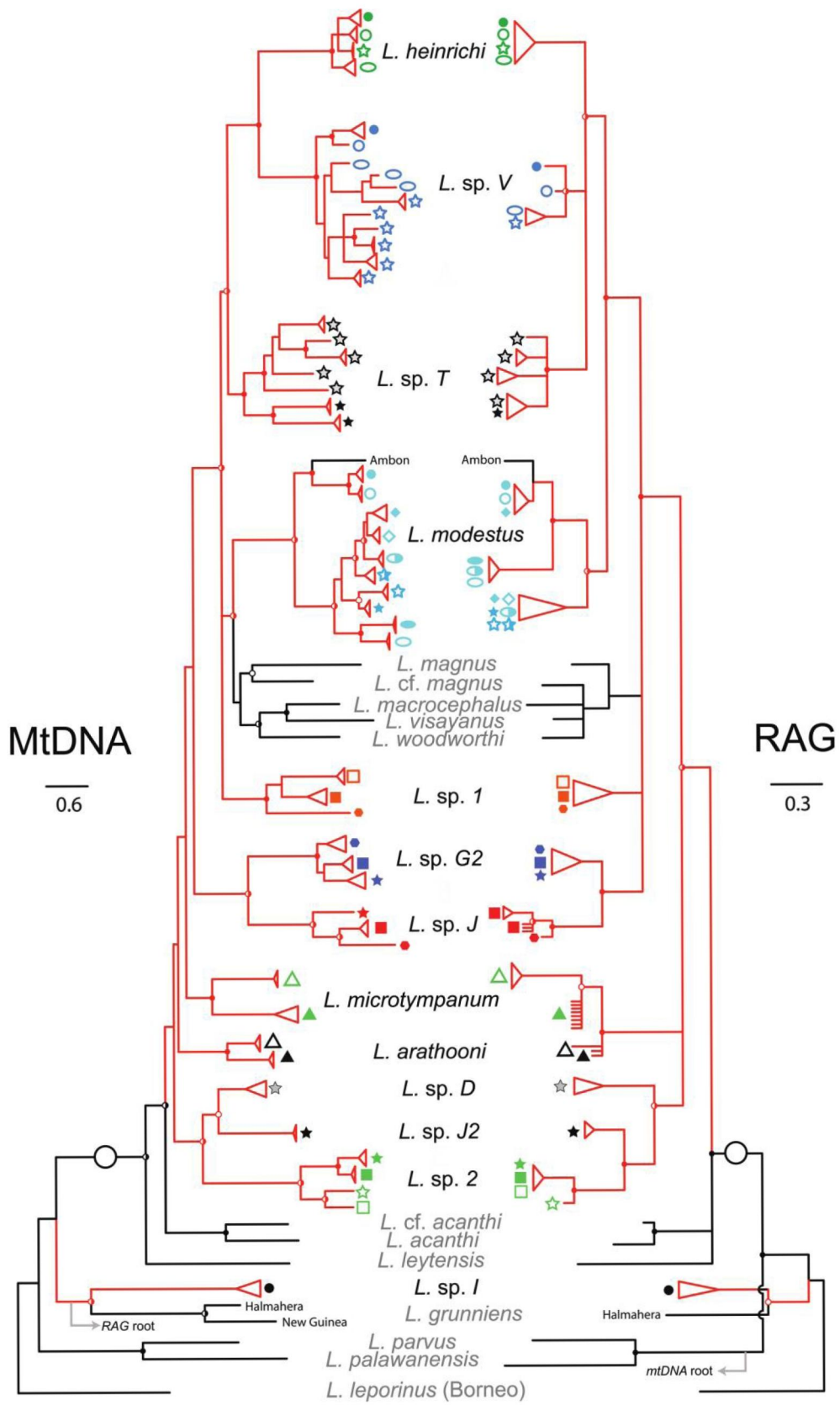
For the BP&P analysis, we used the mtDNA phylogeny depicted in figure 2 to generate a guide tree. Priors and distributions were assigned following recommendations from the authors of BP&P (Yang and Rannala 2010). Specifically, parameters that required priors include the population size parameters for each lineage ( $\theta$  parameters), which received a gamma prior  $G(2, 1,000)$ , and the age of the root of the species tree ( $\tau_0$ ), which was also assigned a gamma prior  $G(2, 1,000)$ ; other divergence times were generated from the Dirichlet distribution (eq. [2] in Yang and Rannala 2010). Because of haploidy and maternal inheritance, the effective population size ( $N_e$ ) of mtDNA is

expected to be approximately one-fourth that of autosomal DNA under ideal expectations. Additionally, mtDNA has a much higher rate of mutation than autosomal DNA. To accommodate these features in the BP&P analysis, we used the “heredity” and the “locusrate” parameters, respectively. The heredity parameter allows different loci to have different heredity scalars with a gamma prior  $G(1.39, 2.22)$  assigned to each locus and the posterior distribution of this scalar estimated from the data. These values were selected in order to specify a mean of 0.625 and standard deviation of 0.530, which is expected if one analyzes two loci with inheritance scalars of 0.25 and 1.0, as expected from the 1:4 ratio of  $N_{e\text{-mtDNA}} : N_{e\text{-aDNA}}$ . In the current version of BP&P, it is not possible to fix these parameter values. The locusrate parameter allows each locus to have a fixed mutation rate scalar; this scalar was calculated on the basis of the Jukes-Cantor-corrected divergence from the outgroup sequence.

Because no more than 100 individuals can be analyzed with the current version of BP&P, we divided our efforts to delimit species of Sulawesi fanged frog into “problem areas,” including (1) *L. arathooni* and *L. microtypanum*, (2) *Limnonectes* sp. V, (3) *Limnonectes* sp. T, and (4) *L. modestus* (fig. 2). For each analysis, we took the following measures to ensure that our results were robust to the priors and other parameter settings: (1) we performed two independent runs with different random seeds, (2) we used both species delimitation algorithms described by Yang and Rannala (2010), (3) we used different parameter values for each speciation delimitation algorithm (algorithm 0,  $\epsilon = 2, 5, 10, \text{ or } 20$ ; algorithm 1,  $\alpha = 1, 1.5, \text{ or } 2$ ; and  $m = 0.5, 1, \text{ or } 2$ ), and (4) we performed runs with only the autosomal data but with a guide tree based on mitochondrial DNA. Runs with all possible combinations of these parameter values were performed on the sharcnet computer cluster (<http://www.sharcnet.ca>). Following recommendations in the BP&P documentation, we adjusted the “fine-tune” parameters of each run so that the acceptance proportions were in the interval (0.15, 0.7). We used the full *RAG1* sequence but only the *COI* sequence for mtDNA because this region was sequenced for all samples in these analyses.

*Characterizing an Adaptive Radiation on Sulawesi.* Adaptive radiations are characterized by rapid diversification of ecologically relevant phenotypes in closely related species. Previous work has established that Sulawesi fanged frogs are closely related to one another although not monophyletic, with *Limnonectes* sp. I being the most divergent from the other species on Sulawesi (Evans et al. 2003c). We tested two predictions that are consistent with adaptive radiation on Sulawesi.

Our first prediction, derived from our observations on



natural history, was that morphological evolution of fanged frogs on Sulawesi and in the Philippines is driven by natural selection for multiple body size optima in sympatric species. Because more *Limnonectes* species occur in sympatry on Sulawesi than in the Philippines, this analysis is dominated by data from Sulawesi. To test this assertion, we took morphological measurements from up to the 10 largest individuals from as many species and from male and female specimens that were available in United States, Indonesian, and Philippine museum collections ( $n = 2-47$ ). We analyzed each sex separately, because many species are sexually dimorphic (table 1); sex was determined by gonadal inspection. We focused on the 10 largest individuals, with the assumption that these specimens best represented the typical maximum adult size. We created chronograms from the Bayesian mtDNA and *RAG1* phylogenies, using r8s, version 1.71 (Sanderson 1997, 2002), by first selecting a representative sequence from each species for which measurements were obtained and then trimming off all other branches. The resulting phylograms were used for r8s cross-validation and chronogram construction. We then used Brownie, version 2.1 (O'Meara et al. 2006), and OUCH (Butler and King 2004; King and Butler 2009) to estimate the likelihood and Brownian motion rate parameter(s) of a model with one rate of size evolution over the fanged frog topology (the BM1 model) and a model with two rates of size evolution, one for Philippine fanged frogs and one for Sulawesi fanged frogs (the BM2 model). Using OUCH, we also explored scenarios of morphological diversification, using Ornstein-Uhlenbeck (OU) processes, following the approach of Butler and King (2004). The first OU model we considered (OU1) has one global optimum for all species on Sulawesi and in the Philippines and their ancestors. The second and the third OU models (OU3 and OU4) assume small, intermediate, or large optima for sympatric species, as in Butler and King (2004). For models OU3 and OU4, species categories from regions with three sympatric *Limnonectes* species (North Sulawesi, West Sulawesi, East Sulawesi, and Mindanao) were based on the relative sizes of the sympatric species in each region. Species from regions with only two sympatric *Limnonectes* species (southwest Sulawesi, Palawan, Luzon, and Visayas) were categorized as either small or large on the basis of their relative sizes in each region,

with the exception of those on Visayas, which were categorized as medium (*Limnonectes leytensis*) and large (*Limnonectes visayanus*) because *L. leytensis* also occurs in sympatry with two other species (one larger and one smaller) on Mindanao. Species from regions with no other *Limnonectes* species (*Limnonectes* cf. *acanthi* on Mindoro and *Limnonectes magnus* from a high elevation on Mindanao) were categorized as large because each was most similar in size to other large *Limnonectes* in the study. In this way, with the exception of the three species that do not co-occur with other *Limnonectes*, the size categories of each species are based only on comparison with other sympatric species and not on phylogenetic relationships among species or on the relative size across all species. The difference between models OU3 and OU4 is that the OU3 model assumes an intermediate optimum for internal branches, whereas the OU4 model assumes a fourth ancestral category. These analyses were performed using log-transformed measurements for each sex and chronograms from each locus (mtDNA and *RAG1*).

The null hypothesis of these models assumes that morphological variation can be explained by Brownian motion (the BM1 model). We used multiple approaches to assess whether the alternative models (BM2, OU1, OU3, and OU4) provided a significantly better fit for the data including (a) a likelihood ratio test, (b) the AIC, (c) the AIC with a correction for small sample size (AICc), and (d) simulations. For the simulations, random deviates from the fitted BM1 model were generated for each sex and for each genealogy (mtDNA and *RAG1*), using OUCH. For each random deviate, size categories (small, medium, or large) for each terminal were determined in the same way as for the observed data (i.e., by categorizing species by size relative to other sympatric species), using custom Perl scripts (available upon request). For each simulation, the two terminals with no sympatric *Limnonectes* (*Limnonectes* cf. *acanthi* and *L. magnus*) and *L. visayanus* was placed in the size category with the lowest absolute difference from other species in that category. The likelihood of each model was then calculated using Brownie and OUCH, and the *P* value was estimated from the rank of the observed difference between the likelihood of the alternative and null hypotheses compared with this difference in 999 random deviates from the fitted null model.

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**Figure 2:** Mitochondrial (mtDNA; *left*) and recombination activating gene 1 (*RAG1*; *right*) genealogies for Philippine and Sulawesi fanged frogs (*Limnonectes* species). Nodes with 100%, >95%, and >90% posterior probability are indicated by filled circles, half-filled circles, and open circles, respectively. Lineages from Sulawesi are in red, and those from the Philippines and elsewhere are in black. Some terminal clades are collapsed and represented by triangles with lengths equivalent to the distance between the ancestral node and the longest branch in the clade. Symbols next to clades reflect molecular variation sampled from locations depicted in figure 3. Scale bars next to each genealogy indicate substitutions per site. A circle over a basal branch indicates the point of consistent rooting between the mtDNA and *RAG1* topologies and also with other studies (Evans et al. 2003; McLeod 2010). An arrow on the mtDNA genealogy indicates where the root is located on the *RAG1* genealogy and vice versa.

**Table 1:** Morphological measurements of Sulawesi and Philippine fanged frogs

	$n_1$	Measured	SVL (SD)		SexD?	Reverse?	$\theta$ estimates from mtDNA		$n_2$
			Females	Males			$\theta_s$	$\theta_\pi$	
Philippine species:									
<i>Limnonectes acanthi</i>	10	19	71.21 (4.99)	67.31 (2.33)	Yes	No	.0012	.0012	2
<i>Limnonectes cf. acanthi</i>	10	20	73.31 (2.80)	78.67 (1.92)	Yes	Yes	.0082	.0082	3
<i>Limnonectes cf. magnus</i>	10	10	85.02 (12.06)	94.52 (10.76)	No	No	.0188	.0188	3
<i>Limnonectes magnus</i>	10	10	69.59 (5.01)	79.61 (9.85)	Yes	Yes	.0003	.0003	3
<i>Limnonectes leytensis</i>	10	20	45.19 (2.36)	43.31 (2.82)	No	No	.0235	.0226	8
<i>Limnonectes macrocephalus</i>	10	20	75.02 (10.81)	98.15 (16.05)	Yes	Yes	.0167	.0156	7
<i>Limnonectes palavanensis</i>	10	13	30.63 (3.09)	26.45 (2.51)	Yes	No	...	...	...
<i>Limnonectes parvus</i>	10	10	26.46 (1.12)	24.25 (1.21)	Yes	No	...	...	...
<i>Limnonectes visayanus</i>	10	20	69.29 (12.86)	68.24 (21.37)	No	No	.0133	.0133	2
<i>Limnonectes woodworthi</i>	10	20	71.72 (3.93)	64.85 (3.52)	Yes	No	.0067	.0055	9
Sulawesi species:									
<i>Limnonectes heinrichi</i>	6	6	69.06 (6.37)	66.63 (7.55)	No	No	.0108	.0136	6
<i>Limnonectes modestus</i>	10	47	61.03 (2.06)	50.94 (2.90)	Yes	No	.0021	.0026	15
<i>Limnonectes microtypanum</i>	10	26	59.27 (6.20)	66.20 (3.15)	Yes	Yes	.0025	.0025	2
<i>Limnonectes arathooni</i>	2	2	30.50 (3.79)	37.59 (1.99)	Yes	Yes	...	...	...
<i>Limnonectes sp. D</i>	10	16	82.95 (31.49)	95.68 (29.59)	No	No	.0042	.0046	4
<i>Limnonectes sp. I</i>	10	24	116.74 (4.03)	134.73 (26.28)	Yes	Yes	.0119	.0106	15
<i>Limnonectes sp. 1</i>	5	5	32.53 (5.74)	35.23 (2.84)	No	No	...	...	...
<i>Limnonectes sp. 2</i>	2	2	35.95 (.40)	47.73 (7.25)	No	No	.0122	.0111	4
<i>Limnonectes sp. G2</i>	10	13	55.05 (7.18)	47.11 (2.56)	Yes	No	.0089	.0117	7
<i>Limnonectes sp. T</i>	7	7	43.08 (2.15)	40.62 (4.86)	No	No	.0438	.0516	7
<i>Limnonectes sp. V</i>	2	2	29.30 (13.20)	36.95 (9.29)	No	No	.0281	.0322	18

Note: Snout-vent length (SVL) in millimeters and standard deviation (SD) of up to the 10 largest individuals ( $n_1$ ) in the sample (Measured). Also indicated is whether there is sexual dimorphism (SexD?) under the assumption of a normal distribution and, if so, whether there is reverse sexual dimorphism, with larger males (Reverse?). Estimates of the population polymorphism parameter  $\theta$  per site from segregating sites ( $S$ ) and average pairwise nucleotide diversity ( $p$ ) are listed with the number of sequences used to estimate these parameters ( $n_2$ ). Some values are missing from this analysis due to a lack of polymorphism data.

Another prediction associated with an adaptive radiation on Sulawesi is that morphological differences between species would be greater when two species were sympatric, after controlling for the effect of genetic relationships. We tested this using partial Mantel tests as implemented by the program PASSaGE, version 2.0, release 7.29 (Rosenberg 2008). The following three matrixes were used in this analysis: (1) morphological distance, (2) the extent of sympatry, and (3) genetic distance. The null hypothesis of this test is that there is no correlation between morphological distance and the degree to which species are codistributed after holding constant the effect of genetic distance. The alternative hypothesis of multiple body size optima in sympatric species proposes that there is more morphological variation among sympatric species than among nonsympatric species after controlling for the impact of evolutionary relationships. We performed separate analyses for frogs from Sulawesi and frogs from the Philippines because we expected larger morphological variation among sympatric species on Sulawesi than among sympatric species in the Philippines on the basis of the lower diversity of other frog lineages from Sulawesi (and thus putatively greater ecological opportunity). For each region, a separate

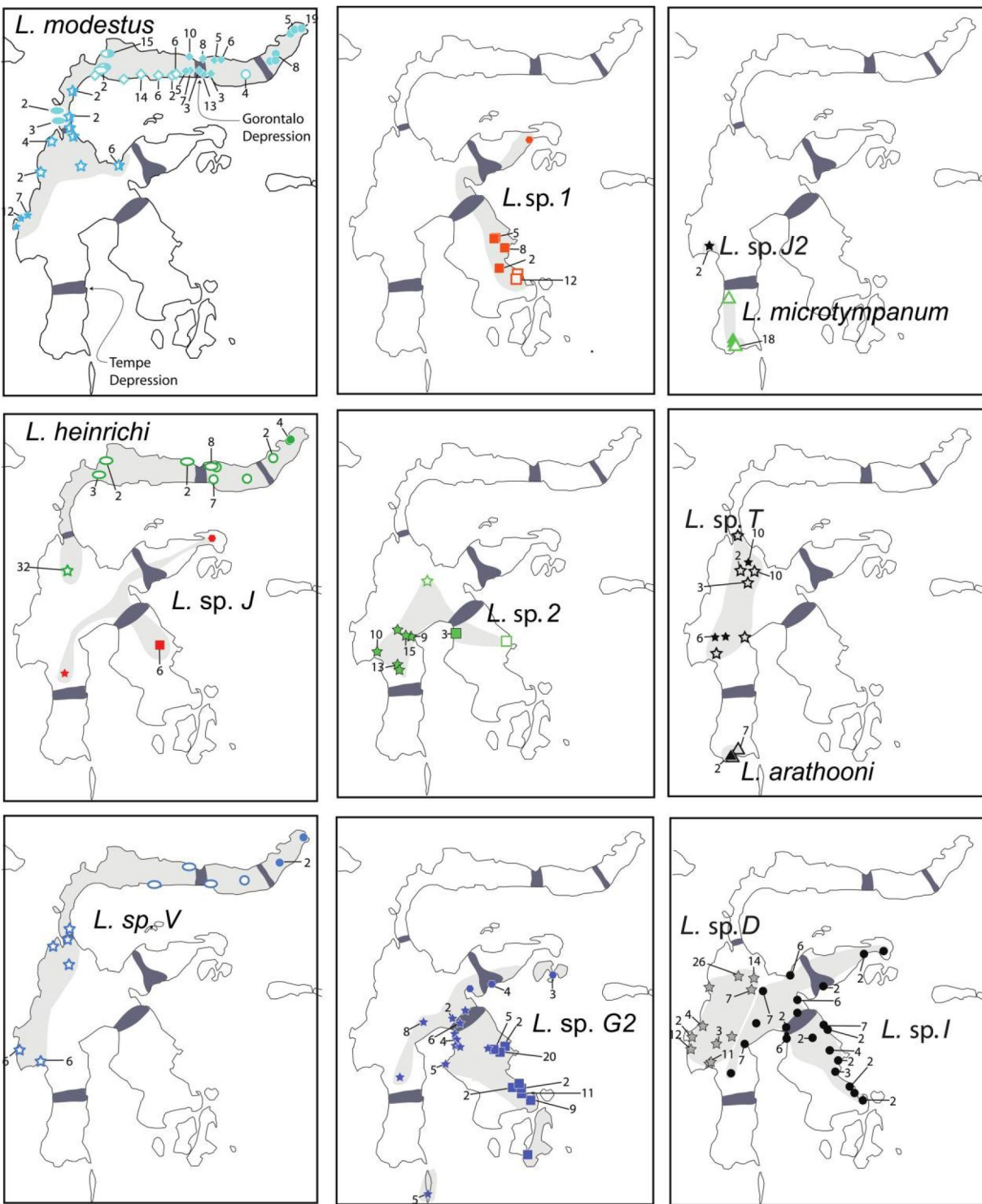
test was performed on each sex to accommodate sexual dimorphism and on each locus to accommodate the impact of genealogical discordance. Morphological distance was quantified as the squared difference in average snout-vent lengths of up to the 10 largest individuals we measured (table 1). The degree of sympatry was quantified as the maximum percentage of range overlap; this was estimated by tabulating pixels from the minimum polygons in figure 3 for Sulawesi and from distributional data in the Philippines from Evans et al. (2003c), using Adobe Illustrator CS2 and Adobe Photoshop CS2 (Adobe Systems). Genetic distance was calculated from the r8s chronograms, using the program PATRISTIC (Fourment and Gibbs 2006).

## Results

### *Species Delimitation and Phylogenetic Relationships*

Relationships among *RAG1* and mtDNA sequences were estimated, using highly parameterized models. For the *RAG1* analysis, a model with a separate GTR + I +  $\Gamma$





**Figure 3:** Ranges of Sulawesi fanged frogs (*Limnonectes* species). Numbers near sampling localities correspond to the number of individuals sampled, and symbols correspond to the variation depicted in figure 1. The locations of the Gorontalo and Tempe depressions are indicated in the *Limnonectes modestus* panel. These regions and other regions indicated in gray correspond to hybrid zones of Sulawesi macaque monkeys.

model for each codon position ( $-\ln L = 2,740.55$ ) was favored over a GTR + I +  $\Gamma$  model for the entire sequence ( $-\ln L = 2,914.91$ ), using a Bayes factors value of 10 (Kass and Raftery 1995); it is reported here. Plots of the post-burn-in posterior distribution of likelihood values, a low average standard deviation of split frequencies (0.0052), and large ( $>100$ ) evolutionarily stable strategy (ESS) values for all parameters suggested that the MCMC chain had converged to a stationary distribution. The mtDNA analysis, in contrast, included many ( $n = 428$ ) unique but often closely related sequences and a biologically appropriate but highly parameterized doublet model for the rDNA stem partition. Even after a long (10 million generations) MrBayes run, the average standard deviation of split frequencies was still  $>0.01$  (it was 0.0188). The ESS of the post-burn-in likelihood scores was  $>100$ , but the ESS values of some parameters, such as the partition rate multipliers and the frequencies of paired bases in the stem regions, were  $<100$  and had a ragged distribution, suggesting poor sampling. However, a plot of the posterior distribution of likelihood did not exhibit an upward trend, analysis of a reduced data set recovered an identical topology, and well-supported relationships in the mtDNA topology depicted in figure 2 are identical to those of previous studies (Evans et al. 2003c; McLeod 2010) and to *RAG1* analyses. This suggests that poor sampling of the posterior distributions of some parameters did not markedly impact our estimation of relationships among species or among diverged clades within species, which is the focus of our interests.

Estimated relationships according to mtDNA data are generally congruent with those estimated with *RAG1* (fig. 2), with the exception of the location of the root and a few relationships that are resolved with poor support in the mtDNA genealogy but unresolved in the *RAG1* genealogy. In the mtDNA phylogeny, the root is in the same location as in the analysis of Evans et al. (2003c), but in the *RAG1* phylogeny, the root is in the same location as the one that was weakly supported by the mtDNA analysis of McLeod (2010). In any case, as indicated in figure 2, with the exception of *Limnometes parvus* and *Limnometes palavanensis*, rooting of relationships among Sulawesi and fanged frogs is consistent between these two loci and also among other studies (Evans et al. 2003c; McLeod 2010). For example, both phylogenies have some Philippine fanged frogs nested within a paraphyletic assemblage of Sulawesi species, supporting the previous proposal that some Philippine fanged frogs arose from an ancestor that dispersed to the Philippines from Sulawesi (Evans et al. 2003c).

Using the genealogical-concordance species criterion for species diagnosis, we identified 12 putative species of fanged frogs on Sulawesi (fig. 2), including two species,

*Limnometes* sp. *J* and *Limnometes* sp. *J2*, that were not sampled in a previous study (Evans et al. 2003c). *Limnometes arathooni* and *Limnometes microtympenum* fail to meet the criterion of monophyly at both loci due to a polytomy in *RAG1*. However, morphology of these species is quite distinct, with *L. microtympenum* being about twice as large as *L. arathooni* (table 1). There is also evidence of different life histories and different advertisement calls for *L. microtympenum* and *L. arathooni* (Brown and Iskandar 2000; R. M. Brown, unpublished data). On the basis of these obvious morphological, ecological, and acoustic differences, we diagnose these lineages as separate species. Thus, the total number of Sulawesi fanged frog species that we recognize here is 13. Species groups on Sulawesi that are supported by *RAG1* and mtDNA include *Limnometes heinrichi* + *Limnometes* sp. *V* + *Limnometes* sp. *T*, *Limnometes* sp. *G2* + *Limnometes* sp. *J*, and *Limnometes* sp. *D* + *Limnometes* sp. *2* + *Limnometes* sp. *J2* (fig. 2). *Limnometes modestus* also occurs on the island of Ambon, and lineages related to *Limnometes* sp. *I* occur in the Moluccas and on New Guinea.

The Bayesian species delimitation analysis (BP&P) supported the recognition of all of the species we tested and further suggested that there are additional species beyond those identified by the genealogical-concordance criterion. The posterior probability of all analyses performed on *L. arathooni* and *L. microtympenum* recovered 99.9%–100% posterior probabilities for each of these species, including the analyses in which the mtDNA guide tree was used with the *RAG1* data only. For *Limnometes* sp. *T*, the BP&P analysis provided strong support for two separate species contained within this lineage; posterior probability of each one was 99.8%–100%, depending on the parameter settings. This species was not further divided by the genealogical-concordance criterion, because the two mtDNA clades did not correspond to the two *RAG1* clades (fig. 2). Analysis with both loci from *Limnometes* sp. *V* supported separate species status for the eastern and western populations (posterior probability, 95.1%–98.9%). However, when the mtDNA guide tree was used for the *RAG1* data alone, one analysis did not support separate species status at the 95% posterior probability level (algorithm 0, smoothing parameter set to 2; posterior probability, 92.4%).

In *L. modestus*, variation in mtDNA and *RAG1* is mostly allopatrically distributed, but gene flow or ancestral polymorphism is suggested by incongruent genealogical relationships among these loci: the margins of the most divergent clades in each of these loci are in different locations (east of the Gorontalo Depression in mtDNA but west of the Gorontalo Depression in *RAG1*; fig. 3). When both loci were analyzed together, BP&P analysis recovered strong support for the recognition of each of the six major

mtDNA clades as separate species (posterior probability = 100% for each clade). When the mtDNA tree was used as a guide for the *RAG1* data alone, strong support (>95% posterior probability) was recovered for dividing *L. modestus* into just two species (one representing the easternmost mtDNA haplotype clade and a second including the other samples).

In summary, the BP&P analysis supported the recognition of *L. arathooni* and *L. microtympanum* as separate species and the further division of *Limnonectes* sp. *T*, *L. modestus*, and possibly *Limnonectes* sp. *V* into pairs of sister species, which brings the total species on Sulawesi up to 16. However, we conservatively follow the diagnosis based on genealogical concordance and morphology at this time because of concerns related to violation of assumptions of the BP&P analysis that are discussed below.

### Species Distributions

The geographically dense sampling analyzed here provides, for the first time, a high-resolution estimate of the ranges of these putative species (fig. 3), which allows us to quantify with precision the degree of sympatry (or lack thereof) for each pair of species. Analysis of new samples presented here substantially extends the ranges of several species, including (a) *L. heinrichi*, which had been previously reported from east of the Gorontalo Depression and which also occurs throughout the northern peninsula and a portion of the west-central region of Sulawesi, (b) *Limnonectes* sp. *V* and *L. modestus*, which had been previously reported on the northern peninsula and which also occur on the west side of the west-central region of Sulawesi; (c) *Limnonectes* sp. *I*, which occurs further west than previously reported but never in sympatry with *Limnonectes* sp. *D*; and (d) *Limnonectes* sp. *2*, which occurs east and north of the previously reported range. The highest diversity of Sulawesi fanged frogs is in Lore Lindu National Park, where at least five sympatric species occur: *L. modestus*, *L. heinrichi*, *Limnonectes* sp. *T*, *Limnonectes* sp. *V*, and *Limnonectes* sp. *D*. Other parts of Sulawesi have three or four species (in the north: *L. modestus*, *L. heinrichi*, and *Limnonectes* sp. *V*; in the west-central area: *Limnonectes* sp. *2*, *Limnonectes* sp. *T*, *Limnonectes* sp. *D*; and in the southeast and east-central areas: *Limnonectes* sp. *I*, *Limnonectes* sp. *G2*, *Limnonectes* sp. *1*, and *Limnonectes* sp. *2*), except the southwest peninsula, where we sampled only two (*L. microtympanum*, and *L. arathooni*). Most Philippine islands, in contrast, support only one or two *Limnonectes* species, the notable exception being portions of Mindanao where up to five species are present, with a maximum of up to four sympatric species observed in one location (Picop,

southeast Mindanao: *Limnonectes* cf. *magnus*, *L. parvus*, *Limnonectes leytensis*, and *Limnonectes feneri*).

Distributions of some species (*L. arathooni*, *L. microtympanum*, *Limnonectes* sp. *D*, *Limnonectes* sp. *T*, *Limnonectes* sp. *J2*) fall within areas of endemism identified on Sulawesi monkeys and toads (Evans et al. 2003b, 2008). Some more widely distributed species (*L. modestus*, *L. heinrichi*, *Limnonectes* sp. *V*) have nonoverlapping mtDNA haplotype clades whose distributions correspond roughly with these areas (figs. 2, 3; Evans et al. 2003b, 2008).

### Adaptive Radiation

To explore the hypothesis that fanged frogs adaptively radiated, we evaluated models of morphological evolution summarized in figure 4. In all of these analyses, the OU3 model was best based on the AIC and AICc (fig. 4; table 2). Some optimal values for each state were extreme, but all were still within the range observed in nature: for the mtDNA chronogram, the ancestral small, intermediate, and large sizes were 25.0, 50.3, and 107.8 mm, respectively, for females and 21.4, 44.5, and 219.4 mm, respectively, for males; for the *RAG1* chronogram, these values were 28.6, 48.1, and 109.9 mm, respectively, for females and 20.7, 49.2, and 151.2 mm, respectively, for males. The OU4 model was also significantly favored over the BM1 model according to all of the statistics except female body size on the mtDNA genealogy, for which simulations did not recover a significant difference ( $P = .060$ ; table 2). The OU model with a single optimum (the OU1 model) was not significantly better than the random-walk (Brownian motion) model for either sex with either genealogy.

Another line of evidence that is consistent with an adaptive radiation of Sulawesi fanged frogs is faster phenotypic evolution on this island compared with other areas. To evaluate variation in rates of morphological evolution, we compared a model in which morphological variation accumulated via Brownian motion at a constant rate in the Philippines and on Sulawesi (the BM1 model) with a model with different rates in each of these regions (the BM2 model). For females (but not males), we found that the model with a different rate of body size evolution for Sulawesi and Philippine frogs was significantly preferred over a model with the same rate on Sulawesi and in the Philippines (table 2). An exception was that the BM2 model was not significantly preferred over the BM1 model on the basis of simulations with the *RAG* genealogy (table 2). When female body size was analyzed on the mtDNA genealogy, using the BM2 model, an ancestral state of 44.3 mm was inferred and the inferred rate on Sulawesi (0.47) was almost six times greater than the inferred rate of evolution in the Philippines (0.08). (These evolutionary rates

**Table 2:** Results of tests for different rates of morphological evolution in Sulawesi and Philippine fanged frogs, using Brownian motion and the Ornstein-Uhlenbeck process

	Females					Males				
	BM1	BM2	OU1	OU3	OU4	BM1	BM2	OU1	OU3	OU4
mtDNA phylogeny:										
lnL	-10.42	-7.94	-10.98	3.52	3.62	-10.65	-10.27	-11.66	4.29	4.76
AIC	24.83	21.9	27.96	2.97	4.75	25.29	26.55	29.33	1.43	2.48
AICc	25.50	23.3	29.38	6.97	10.75	25.96	27.96	30.74	5.43	8.48
df	2	3	3	5	6	2	3	3	5	6
<i>P</i> value:										
$\chi^2$	...	.026	1.000	<.001	<.001	...	.387	1.000	<.001	<.001
Simulations	...	.037	.713	.036	.060	...	.456	.882	.012	.028
Comments	...	BP	BP	BP	...	...	BP	BE	BE	...
RAG1 phylogeny:										
lnL	-9.96	-7.58	-10.18	7.58	7.73	-9.83	-9.71	-10.65	7.81	9.81
AIC	23.92	21.17	26.36	-5.16	-3.45	23.65	25.42	27.31	-5.61	-7.63
AICc	24.58	22.58	27.77	-1.16	2.55	24.32	26.83	28.72	-1.61	-1.63
df	2	3	3	5	6	2	3	3	5	6
<i>P</i> value:										
$\chi^2$	...	.0293	1.000	<.001	<.001	...	.63028	1.000	<.001	<.001
Simulations	...	.066	.618	.002	.005	...	.695	.795	.005	.005
Comments	...	...	BP	BP	BP	...	...	BP	BE	BE

Note: Model acronyms are explained in text and illustrated in figure 3. *P* values of the more parameterized model were assessed, using a  $\chi^2$  test based on comparison with the model with one Brownian motion parameter and using simulations as described in “Methods”; individually significant values are indicated with italic type. Additionally, for each model, the Akaike Information Criterion (AIC), an AIC modified for small sample size (AICc), and degrees of freedom (df) are listed. Tests were performed, using a chronogram estimated from mitochondrial DNA (mtDNA) and from an autosomal gene, the recombination activating gene 1 (*RAG1*). For Ornstein-Uhlenbeck models, comments refer to estimated optimal size and are either biologically plausible (BP) if it was  $\pm 10\%$  of observed body sizes or biologically extreme (BE) if at least one optimum was substantially different from the observed body sizes but still within the range of body sizes of extant frogs. Biologically implausible optima were not recovered in any of these analyses.

refer to morphological change over time; however, the units of time are not known because we do not have a suitable calibration point for the phylogenies used in this analysis.) When the *RAG* genealogy was used, the results were similar: the BM2 model estimated an ancestral state of 46.1 mm and the rate on Sulawesi (0.54) was much faster than the inferred rate in the Philippines (0.10). Although not significantly different, the BM2 model also inferred a faster rate of evolution of male body size on Sulawesi than in the Philippines, using both genealogies. Taken together, the results support (1) multiple size optima for sympatric fanged frogs on Sulawesi and in the Philippines and (2) a faster rate of morphological evolution for female fanged frogs on Sulawesi than in the Philippines.

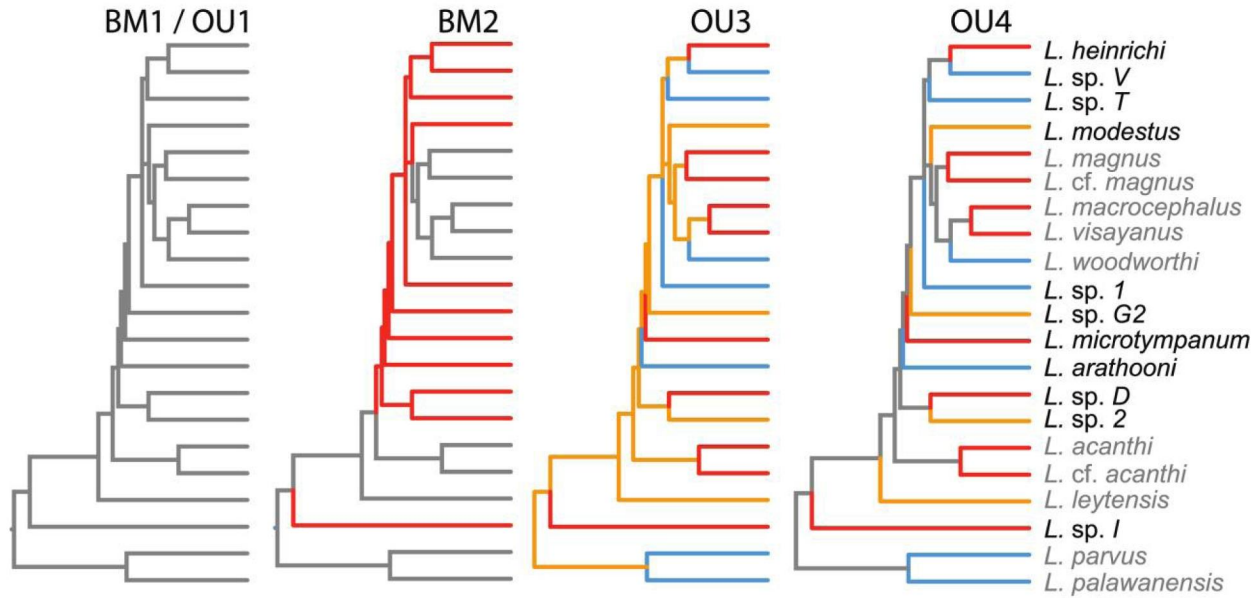
Under the hypothesis of an adaptive radiation, we also predicted that morphological divergence would be higher among sympatric species than among allopatric species on Sulawesi and that this correlation should be stronger among fanged frogs from Sulawesi than among those from the Philippines. Consistent with this expectation, a partial Mantel test recovers a significant positive correlation between morphological divergence and sympatry for both sexes on Sulawesi when the effects of genetic distances from mtDNA or from *RAG1* are held constant (table 3).

The partial correlation was not significant for either sex in Philippine fanged frogs, using genetic distances from either chronogram (table 3). In pairwise comparisons between species, our distributional data indicate that Sulawesi has at least 21 sympatric species pairs, with a maximum range overlap of 3%–96%. Morphological variation is perhaps lowest on the northern peninsula, where *L.*

**Table 3:** Results of partial correlation tests for a relationship between size variation and sympatry on Sulawesi and in the Philippines

Genealogy	Females		Males	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Sulawesi <i>Limnonectes</i> :				
mtDNA	.2241	.0330	.3505	.0020
<i>RAG1</i>	.4163	.0040	.4668	.0040
Philippine <i>Limnonectes</i> :				
mtDNA	.1858	.1080	.0933	.2210
<i>RAG1</i>	.2058	.1150	.1232	.2240

Note: The partial correlation coefficient (*r*) and the *P* value of the null hypothesis of no correlation are shown. Individually significant values are indicated with italic type. mtDNA, mitochondrial DNA; *RAG1*, recombination activating gene 1.



**Figure 4:** Models of morphological evolution evaluated for Sulawesi and Philippine fanged frogs, including Brownian motion with one (BM1) or two (BM2) rate parameter(s) and the Ornstein-Uhlenbeck process with one (OU1), three (OU3), or four (OU4) optima. The BM2 model has different rates of Brownian motion for Sulawesi and Philippine species. The OU3 and OU4 models have small (blue), medium (orange), and large (red) optima, and the OU4 model has an additional ancestral optimum (gray). Shown is the mtDNA phylogeny with male morphological categories; similar models were considered for the mtDNA phylogeny with female morphology, for the recombination activating gene 1 (*RAG1*) phylogeny with female morphology, and for the *RAG1* phylogeny with male morphology.

*heinrichi* is only slightly larger than *L. modestus*, but this could be because these species are also recently diverged (fig. 2). Other parts of Sulawesi have more pronounced variation in size among sympatric species, even among closely related species. For example, *Limnonectes* sp. *D* and *L. heinrichi* are large, whereas their close relatives *Limnonectes* sp. 2 and *Limnonectes* sp. *V*, respectively, are sympatric but small. The two largest species, *Limnonectes* sp. *I* and *Limnonectes* sp. *D*, are not closely related, but our extensive field collections indicate they are also never found in sympatry (fig. 3). Two closely related species with small males, *Limnonectes* sp. *V* and *Limnonectes* sp. *T*, are almost entirely allopatric.

Our morphological measurements from Philippine fanged frogs included only eight sympatric species pairs. Six of these occur on Mindanao, where Philippine fanged frog diversity is highest. The other two include one morphologically diverged pair of species (*L. palawanensis* and *Limnonectes acanthi*) that are evolutionarily diverged, and one morphologically similar pair of species (*Limnonectes macrocephalus* and *Limnonectes woodworthi*) that are closely related (fig. 2).

As an alternative to an adaptive radiation, another explanation for the higher variation in body size of Sulawesi fanged frogs is that their population sizes might be smaller

than those of the Philippines species, resulting in greater morphological variance on Sulawesi due to genetic drift. To explore this possibility, we estimated  $\theta_s$  and  $\theta_\pi$  from homologous mtDNA sequences from rDNA genes from sequence data reported by Evans et al. (2003c). We used this data set instead of the new data presented here because the former data set has population sampling of Sulawesi and Philippine species from the same loci. Both  $\theta$  estimates tended to be larger in Sulawesi species than in Philippine species (table 1), suggesting that morphological variation due to genetic drift should actually be higher in the Philippines than on Sulawesi if the mutation rate and selection pressure on these loci are similar in both regions. Additionally, many of the Sulawesi estimates of  $\theta$ , such as for *L. modestus*, *Limnonectes* sp. *V*, and *L. heinrichi*, are probably underestimates because data were not collected by Evans et al. (2003c) for the entirety of the ranges of these species. We were missing molecular polymorphism data from two Philippine and two Sulawesi species that were included in the morphological analysis. Two Philippine species (*L. palawanensis* and *L. parvus*) and one Sulawesi species (*Limnonectes* sp. *I*) have relatively large ranges, and one species (*L. arathooni*) has a small range. Overall, then, we conclude that the hypothesis of genetic drift as the

main driver of morphological divergence of Sulawesi and Philippine fanged frogs is inconsistent with our data.

### Discussion

In this study, we synthesized information from multiple sources to estimate species diversity of fanged frogs on Sulawesi and to test for evidence of an adaptive radiation. We provide support for at least 13 species of fanged frog on Sulawesi, nine of which do not have formal species descriptions. We find that female fanged frogs had a significantly faster rate of body size diversification on Sulawesi than in the Philippines and that morphological variation was significantly higher among sympatric than nonsympatric species on Sulawesi but not in the Philippines. We also find that a model with multiple ecological optima for sympatric species is significantly supported over alternative models without ecological optima. These results, combined with field observations of other phenotypic variation associated with microhabitats (variation in the extent of webbing and the presence of derived reproductive modes), suggest that fanged frogs underwent a remarkable adaptive radiation on Sulawesi.

To take an initial step toward species diagnosis, we applied the genealogical-concordance criterion to phylogenies estimated from two molecular markers, and we also considered morphological data. The genealogical-concordance criterion potentially could overdiagnose species diversity in situations when population structure or incomplete sampling causes reciprocal monophyly of multiple markers within a species. It could also underdiagnose species diversity if speciation occurred so recently that ancestral variation has not sorted into reciprocally monophyletic groups in sister species. Application of the genealogical-concordance criterion resulted in the identification of 12 hypothesized species of fanged frogs on Sulawesi, and consideration of morphological, ecological, and acoustic information supports the delineation of *Limnonectes arathooni* and *Limnonectes microtympanum*, suggesting a total of at least 13 fanged frog species on Sulawesi. The substantial genetic and morphological divergence between most putative species identified here (table 1; fig. 2; data not shown) allows us to dismiss overdiagnosis as a possibility; we thus view the number of fanged frog species estimated here ( $n = 13$ ) as a minimum or a conservative estimate.

A Bayesian method for species delimitation (BP&P) supports the recognition of *L. arathooni* and *L. microtympanum* and suggests that *Limnonectes modestus*, *Limnonectes* sp. *T*, and potentially *Limnonectes* sp. *V*, as identified by the genealogical-concordance criterion, are actually species complexes. However, the BP&P assumption of panmixia is clearly violated by some of these species. *Lim-*

*nonectes modestus* and *Limnonectes* sp. *V* span multiple areas of endemism (Evans et al. 2003b) that roughly correspond to allopatric intraspecific mtDNA clades. *Limnonectes arathooni*, *L. microtympanum*, and *Limnonectes* sp. *T* have fairly small ranges restricted to areas of endemism on Sulawesi, although isolation by distance probably operates. At this time, therefore, we cannot distinguish between the possibilities that (1) these widespread species actually represent multiple recently diverged lineages or (2) they are in fact single species exhibiting the effects of isolation by distance and vicariance. We suspect that future field studies, more focused population demographic analyses with multilocus data, study of museum specimens, and characterization of other data types, including vocalization, voicelessness, and ecology, will uncover additional fanged frog species on Sulawesi or provide support for further division of the species diagnosed here. In any case, it is remarkable that species diversity of fanged frogs on Sulawesi is similar to that in the Philippines, even though Sulawesi is much smaller and less fragmented. These similar levels of diversity contrast sharply with those of other frog genera, which are either more diverse in the Philippines or simply not found on Sulawesi (Alcala and Brown 1999; Inger 1999; Brown 2007), and they contradict the expectations of island biogeography that larger landmasses should host higher species diversity.

### Adaptive Radiation

An adaptive radiation is the rapid evolution of phenotypic diversity that is correlated with the environment in a way that is relevant to fitness. The difference between nonadaptive diversification and adaptive radiation is somewhat qualitative. Adaptive radiations are generally more spectacular in the sense that diversification is quicker and accompanied by more pronounced phenotypic change in response to the environment (Kassen 2009; Glor 2010; Losos 2010). Of course, not all radiations must be adaptive: diversification could be driven primarily by vicariance without substantial ecological or phenotypic diversification (Gittenger 1991; Kozak et al. 2006; Rundell and Price 2009). Consistent with this, similarities in species distributions and population structure argue that differentiation of many endemic terrestrial vertebrates on Sulawesi was triggered by habitat fragmentation. For example, several endemic groups (e.g., Sulawesi macaque monkeys, Celebes toads) exhibit a surprisingly similar distribution of molecular variation (Evans et al. 2003a, 2003b, 2004, 2008), and the ranges of *Limnonectes* sp. *V* and *Limnonectes* sp. *I* bear striking similarities to those of the flying lizard species *Draco spilonotus* and *Draco beccarii*, respectively (McGuire et al. 2007). Ranges and population structures of other taxa, such as tarsiers (Merker et al.

2009; Shekelle et al. 2010), bats (Campbell et al. 2007), and tree frogs (Brown et al. 2010), also share aspects of these patterns (Evans 2011). However, in sharp contrast to these other groups, Sulawesi fanged frogs also include multiple species living in sympatry, an observation that suggests that habitat fragmentation was not the only factor that drove their diversification.

Here we provide statistical support for some of the criteria laid out by Schluter (2000b) for an adaptive radiation, including recent common ancestry, comparatively rapid diversification, and natural selection for multiple phenotypic optima in sympatric species. By analyzing morphological data from *Limnonectes* species in a phylogenetic context, we found that a model of evolution with multiple body size optima for sympatric species was favored (table 2), and this significant result is reinforced by simulations. The main point of performing these simulations was to explore the effect of the seemingly circular approach of using observed relative body sizes of sympatric species to generate the OU models with ecological optima on lineages leading to each species. The simulations suggested that this approach was slightly counterconservative in that simulated *P* values were higher than those based on the AIC and the AICc (table 2). The effect was small, however, and each of these metrics always supported the OU3 model over the BM model and almost always supported the OU4 model over the BM model.

There are multiple possible interpretations of these findings. Our null hypothesis of morphological divergence by Brownian motion is potentially consistent with neutral divergence by genetic drift, with divergence by drift coupled with directional selection, where the direction of selection changes over time, or with neutral evolution, within a constrained morphospace (Lande 1976; Felsenstein 1988; Harmon et al. 2010). Butler and King (2004) invoked the concept of character displacement to account for significant improvement of a multiple-optima OU model over a BM model in an analysis of body size of sympatric lizards (*Anolis*). Character displacement occurs when species evolve in opposite directions in a zone of sympatry but occupy similar ecological niches in zones of allopatry (Brown and Wilson 1956; Schluter and McPhail 1992; Taper and Case 1992; Losos 2000; Schluter 2000a). Although a period of allopatric divergence followed by range expansion and secondary contact is possible for Sulawesi *Limnonectes*, it is also conceivable that geographically codistributed and closely related species (e.g., *Limnonectes heinrichi* and *Limnonectes* sp. V or *Limnonectes* sp. G2 and *Limnonectes* sp. J) evolved in situ. Ecological release in response to ecological opportunities of the depauperate amphibian community on Sulawesi could have precipitated ancestral niche expansion, microhabitat specialization, and speciation.

A key characteristic of an adaptive radiation is that diversification should be rapid (Schluter 2000b; Glor 2010). We lack fossil or geological information with which to calibrate a molecular clock, so we did not attempt to provide a temporal context for the diversification of Sulawesi fanged frogs. Instead, we relied on a comparison to Philippine fanged frogs, which represent an equally aged or older radiation on a larger habitat, to argue that morphological diversification on Sulawesi was comparatively rapid and more diverse than expected on the basis of habitat size. In females, a model with a faster rate of morphological evolution on Sulawesi than in the Philippines was preferred over a model with the same rate of morphological evolution in both regions. A potential caveat is that the data best fit the OU3 model, and so faster rates of evolution should therefore be inferred for younger clades (Harmon et al. 2010). However, Sulawesi *Limnonectes* are generally not more recently diverged than the Philippine species (fig. 2), so the inference of faster rates of evolution in female *Limnonectes* does not appear to be an artifact of the better fit of the OU3 model. Other biases could be introduced by missing data or oversimplification of the number of optima in each suite of sympatric species. Because missing data for the morphological analysis were mostly from species that either were rare or had small distributions (or both), we suspect that the impact of missing data is minor. Admittedly, the favored model (OU3) is still coarse and fails to capture realistic aspects of diversification such as a dynamic number of optima through time, dynamic optimal values of these optima through time, and variable influences of biogeography and competition through time.

While controlling for evolutionary relationships with partial Mantel tests, we also found significantly higher morphological diversity among sympatric species than among allopatric species on Sulawesi but not in the Philippines. Here we permuted residuals, as suggested by Legendre (2000), although it is unclear whether our relatively small sample size ( $n = 11$  Sulawesi species and  $n = 10$  Philippine species) could have elevated Type I error (i.e., inappropriate rejection of the null hypothesis; Legendre 2000; Harmon and Glor 2010). Another possible caveat is the observation that frogs may have indeterminate growth (e.g., Baugh and Ryan 2009). However, for most frog species, evidence for indeterminate growth is lacking, juvenile growth greatly exceeds adult growth, and adult body size falls within a narrow range, suggesting that growth ceases or mortality is age dependent (Duellman and Trueb 1994).

Our field observations provide anecdotal support for Schluter's (2000b) third and fourth criteria of phenotype-environment correlation and trait utility. Small frogs with reduced interdigital webbing of the foot and pronounced

subarticular tubercles (an indication of specialization for terrestrial microhabitats) were collected in leaf litter near rivers, medium-sized frogs were collected in close association with rivers with moderate flow rates, and large-sized frogs were observed only in large rivers. Large species (*Limnonectes* sp. *I*, *Limnonectes* sp. *D*, *L. microtypanum*, and *L. heinrichi*) have relatively more extensive interdigital webbing and less pronounced subarticular tubercles compared with the other species; these are universally assumed adaptations to aquatic habitats (Duellman and Trueb 1994; Stebbins and Cohen 1997). *Limnonectes* sp. *I* individuals lack vocal sacs, which conceivably is related to the high-intensity ambient noise of their habitat (Wells 2007). Most strikingly, smaller-bodied fanged frogs have derived modes of reproduction, including parental care in *L. arathooni* (Brown and Iskandar 2000) and *Limnonectes* sp. *T* (D. T. Iskandar, unpublished observations) and viviparity in *Limnonectes* sp. *V* (Iskandar and Tjan 1996), adaptations that facilitate terrestrial reproduction.

We used rDNA sequence data to estimate the population genetic parameter  $\theta$  in order to assess the impact of genetic drift on morphological evolution of fanged frogs from Sulawesi and the Philippines. This could be problematic if molecular variants in this region of the mtDNA were mildly deleterious, because larger effective population size might actually have lower polymorphism, rather than vice versa. While there was no obvious trend suggested by this analysis, clearly a better source of information on  $\theta$  would be silent sites in multiple regions of the genome that are distant from other loci (e.g., Singh et al. 2007); however, this type of data was not available here.

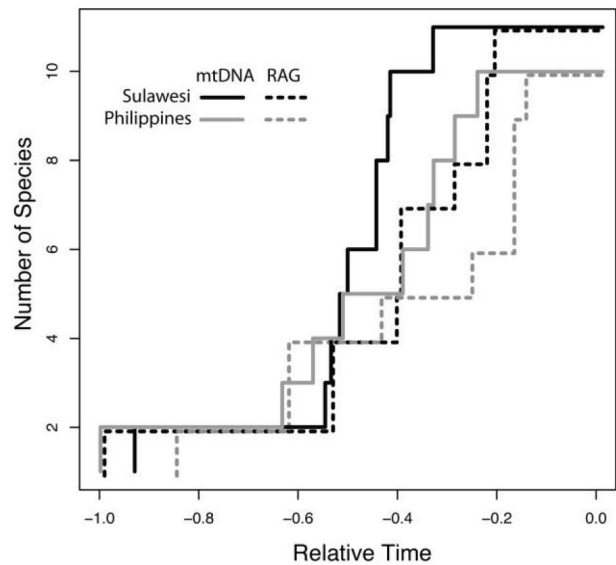
Our use of such a conservative criterion for species diagnosis could have led to pooling of information from multiple distinct species, which could potentially impact our conclusions. For example, if additional species with body sizes intermediate to those detailed here were identified on Sulawesi, this would decrease support for the hypothesis of an adaptive radiation. However, the phylogeography of molecular variation (figs. 2, 3) and BP&P analysis suggests that further species diagnoses would generally divide species identified here into nonsympatric forms, as opposed to teasing apart divergent lineages living in sympatry. For this reason, we do not anticipate that further taxonomic revisions will radically alter our conclusions.

#### *Constraint and Ecological Opportunity*

In theory, adaptive radiations do not go on forever. At some point, an equilibrium is reached, with available ecological niche space becoming saturated (e.g., food resources or physical space becomes limiting), causing diversification to level off because the rate of speciation

equals the rate of extinction (Rabosky 2009). A recent analysis of the iconic adaptive radiation of anolis lizards in the Caribbean, for example, concluded that diversity on three out of four large islands had reached equilibrium: no further adaptive radiation is anticipated (Rabosky and Glor 2010). In contrast, the anticipated decline in rates of diversification of a mature radiation of Philippine shrews may have been offset by a particularly active geological history (Esselstyn et al. 2009). In the Philippines and on Sulawesi, rates of recent speciation of fanged frogs also have not decreased markedly (fig. 5). While the question of whether there is statistical evidence for decreased rates of speciation is an interesting one, we did not attempt here to fit these data to equilibrium and nonequilibrium models because of our small sample size. It is certainly possible that some portions of this radiation have reached species diversity equilibrium while others have not.

While our findings provide support for adaptive radiation on Sulawesi, this is not to suggest that fanged frogs in the Philippines did not adapt to their environment. On the contrary, our fieldwork in the Philippines also suggests a correlation between body size and ecology, with Philippine fanged frogs occupying similar or more general ecological niches (relative to Sulawesi fanged frogs). The large-bodied Philippine fanged frogs *Limnonectes acanthi*, *Limnonectes* cf. *acanthi*, *Limnonectes ferneri*, *Limnonectes macrocephalus*, *Limnonectes magnus*, *Limnonectes* cf. *magnus*, and *Limnonectes visayanus* are found in medium to



**Figure 5:** Number of species through time for Sulawesi and Philippine fanged frogs on the basis of chronograms constructed from mitochondrial DNA (mtDNA) and recombination activating gene 1 (RAG1).



large rivers, whereas the medium- to small-bodied species *Limnonectes leytensis*, *Limnonectes parvus*, and *Limnonectes palavanensis* are found in swamps, seeps, and wide, slow-moving streams. There is circumstantial evidence that in some parts of the Philippines, niches analogous to those occupied by small terrestrial *Limnonectes* on Sulawesi (*L. arathooni*, *Limnonectes* sp. V, *Limnonectes* sp. T, *Limnonectes* sp. I) are occupied by different frog genera or other distantly related *Limnonectes* with similar, independently evolved adaptations. In particular, just like small-bodied fanged frogs on Sulawesi, Philippine *Platymantis* lack webbing, possess protuberant subdigital tubercles, and have a derived reproductive mode (direct development), and some congregate loosely around water (Alcala and Brown 1998, 1999). *Platymantis* diversity on Mindanao, where diversity of Philippine *Limnonectes* is highest, includes only four of the 29 described *Platymantis* species from the Philippines (Frost 2010). *Limnonectes parvus*, the small-bodied fanged frog on Mindanao, has clutches with relatively few large eggs, both of which are correlated with a derived reproductive mode (R. M. Brown, personal observation; Brown and Alcala 1982). Even more striking is the observation that *Platymantis* does not occur on Palawan (Alcala and Brown 1999), which instead is inhabited by the smooth guardian frog *L. palavanensis* (which, as currently recognized, also occurs on Borneo). This species has independently evolved reduced webbing and a derived reproductive mode (egg guarding and tadpole carrying), similar to small terrestrial *Limnonectes* on Sulawesi. In contrast, on Luzon, diversity of Philippine *Platymantis* is high but there are no small terrestrial *Limnonectes*, and on northern Luzon, there is only one *Limnonectes* species (*L. macrocephalus*), which is large bodied. Similarly, *Platymantis* also occurs on Peleng Island (just east of Sulawesi; fig. 1), and no semiterrestrial *Limnonectes* are known from Peleng. Thus, a prominent feature of the adaptive radiation of fanged frogs on Sulawesi is the invasion of a semiterrestrial niche. This ecological conquest was associated with multiple independent reductions in body size, reductions in interdigital webbing, the origin of prominent subarticular tubercles, and multiple independent innovations in reproductive strategies (viviparity and parental care). Arguably, the adaptive radiation of *Limnonectes* also involved portions of the Philippines (Palawan and Mindanao), although in these regions key adaptations (reduced body size, derived reproductive mode) may have evolved in an ancestor on Borneo before dispersal to the Philippines. Another conspicuous feature of the adaptive radiation on Sulawesi was invasion of the fast-moving-water niche, a habitat that favored large body size and more complete interdigital webbing.

Ecological opportunity arises through the appearance of new resources, the extinction of species that previously

used resources, or novel access to resources through phenotypic evolution or dispersal (Simpson 1953; Losos 2010). When ancestors of Sulawesi fanged frogs reached Sulawesi, they encountered unoccupied microhabitats in an isolated and fragmented landscape (Hall 2001). Natural selection for multiple body-size optima favored specialization through speciation, culminating in an adaptive radiation of suites of closely related, morphologically distinct sympatric species in different parts of this island. In nature, few examples exist of species assemblages with replicated sets of convergently evolved phenotypes, a phenomenon known as species-for-species matching (Schluter 1990; Glor 2010; Losos 2010), and in that sense, this discovery on Sulawesi is particularly exciting. This is perhaps more apparent because ecological opportunities on Sulawesi were exploited by closely related species (Mahler et al. 2010). A striking distinction from prominent adaptive radiations in the Caribbean, Hawaii, and the Galapagos Islands (Losos et al. 1998; Grant 1999; Gillespie 2004) is that on Sulawesi, multiple suites of sympatric species independently radiated on only one island. However, this distinction ignores the context of the geological record, which indicates that Sulawesi was formerly an assemblage of several palaeoislands (Hall 2001), and it is possible that the *Limnonectes* adaptive radiation was initiated on this archipelago. Other examples of diverse taxa with multiple sympatric species occur on Sulawesi, such as shrews (Ruedi et al. 1998; Esselstyn et al. 2009) and bent-toed geckos (*Cyrtodactylus*); these would be interesting focal groups for further research on adaptive radiation on this island.

Ecological opportunity is by no means a guaranteed trigger for adaptive radiation (Losos 2010). However, standard island biogeographical theory fails to explain why species diversity of fanged frogs on Sulawesi is similar to rather than lower than that observed in the Philippines, a larger landmass, or why key, ecologically relevant innovations repeatedly arose in closely related species in different geographic regions of Sulawesi. Tellingly, innovations associated with semiterrestriality on Sulawesi are actually not truly novel in the sense that they also evolved independently in other frog lineages in the Philippines (*Platymantis*) or distantly related *Limnonectes* species from the Philippines that occur where *Platymantis* species diversity is low (Mindanao) or absent (Palawan). Together, these observations thus argue that a role of ecological opportunity in speciation is not as unusual as the high endemism of Sulawesi fanged frogs would suggest.

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