Title
Mechanisms of Lineage Divergence in the Radiation of Fanged Frogs (Genus: Limnonectes)

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Mechanisms of Lineage Divergence in the Radiation of Sulawesi Fanged Frogs (Genus: *Limnonectes*)

By

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requirements for the degree of

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of the

University of California, Berkeley

Committee in charge:

Professor Jimmy A. McGuire, Chair
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Professor Ian J. Wang

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ABSTRACT

Mechanisms of Lineage Divergence in the Radiation of Sulawesi Fanged Frogs
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In the 300 million years since the divergence of anurans and caudates, frogs have come to represent nearly 90% of all amphibians and (with the exception of Antarctica) occupy most of the major landmasses on Earth. The range of ecological specializations and phenotypes characteristic of frogs facilitated their colonization of every major habitat type from deserts to rainforests. Given current global ecological conservation concerns, there is an ever-increasing need to assess the drivers of radiative herpetological diversity in areas of especially high species richness and endemism. One such hotspot of global endemism and diversity is the Indonesian island of Sulawesi. Though the biological significance of Sulawesi taxa was noted by Alfred Russel Wallace as far back as the 1800's, in recent years, investigators have not only identified a preponderance of new species on Sulawesi, but several assemblages representing remarkable radiations of novel species.

Herein, I present research that focuses on a recently discovered Sulawesi radiation: that of the so-called 'fanged frogs' of genus *Limnonectes*. This poorly studied assemblage likely includes ~40 species, though only five of have been formally described due to unresolved degrees of morphological, ecological, and molecular disambiguation across species. Very little is known about Sulawesi fanged frog natural- and life histories; thus, the research presented in this compendium explores the ecological mechanisms that facilitated the Sulawesi *Limnonectes* radiation in effort to counterbalance the current dearth of baseline knowledge about the species comprising this assemblage. To accomplish this objective, I conducted extensive field work across the island and subsequently amalgamated molecular, morphometric, behavioral, physiological, and ecological research applications to characterize differential niche use, reproductive biology, and lineage divergence. In Chapter 1, I describe and diagnose a new terrestrially-nesting *Limnonectes* species from South Sulawesi. In Chapter 2, I explore cryptic speciation and highlight the discovery of replicate elevational speciation events in a locally sympatric cohort of fanged frog eco-morphs. In Chapter 3, I characterize the eco-physiological drivers of niche partitioning as they apply to cutaneous water loss, behavioral hydro-regulation, and desiccation tolerance. This body of work advances our understanding of Sulawesi fanged frog natural history and underscores the utility of integrative biological research applications for the purposes of determining the interplay between speciation mechanisms and ecological interactions.

This work is dedicated in honor of my family. I am truly grateful for their unwavering love and support, during my fifteen-year journey pursuing higher education.

Mom, Dad, and Amanda – thank you from the bottom of my heart for always being my biggest cheerleaders and offering boundless encouragement, especially from the first instance of an eight-year-old vehemently proclaiming that his ultimate dream was to become a herpetologist.

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First and foremost, I must thank my family: Valerie Frederick, Thom Frederick, and Amanda Frederick, for their unbridled support. It was a slippery slope once I got my first taste of field work during my undergraduate studies. They've taught me so much about the meaning of unconditional love – by passionately rooting for me as the years wore on and the field stints away from home grew longer and longer, taking me farther and farther away each time. This also goes for my niece and nephew, Abram Maxwell and Dahlia Stephan whom I love endlessly.

Secondly, and of no less importance, I can't proceed without fumbling through an attempt to express the amalgamation of respect, gratitude, and admiration I have for my fearless leader, Jim McGuire. It really is mind-boggling how we got here… From a professor, museum curator, and brilliant herpetologist who was just itching for someone to take up the mantle of frog research in Indonesia, and a cold call from a completely random master's student in Alaska who worked on mountain goats – it really is wild how far we've come. Relaying the degree to which I am continually and utterly inspired by Jim is probably the most conceptually difficult aspect of this dissertation. He is undoubtedly the most supportive advisor one could ever hope for, and (sorry, Amanda, Ross, and Ben!) there is no other human alive that I would rather trudge through the literal muck with- in search of that next great frog discovery than Jim. It goes without saying that he's made me a better scientist, a thinker, writer, and researcher; however, his ability to also thread the needle between exercising patience while also relishing in being my partner in arguments is really something to behold. I'm so incredibly lucky to have been his student and humbled by his unthinkable willingness to approve of me digging into five other dissertations-worth of side projects – a decision that he knows full-well will keep us inevitably locked-in to doing research together for the next decade or three.

I'd also like to express my most sincere gratitude and admiration to the esteemed members of my graduate committee. I can't count the number of crazy looks I've received from fellow students when I've mentioned that I lobbied Grad. Div. to grant me a five-member committee (in contrast to the three-member standard). …That is, of course, until I listed off the utter eminence of professors that have graciously guided me throughout my Ph.D. studies: Eileen Lacey, Marvalee Wake, Ian Wang, and Caroline Williams. I'm forever indebted for the opportunity to have been advised by each of them, and for the care they needlessly took in lending me invaluable guidance over the years. Caroline Williams was pivotal to the development of my ongoing pursuits in the field of physiology. I'm extremely grateful for her insight and limitless well of knowledge on ectotherm-, thermal-, and eco-physiology. To this day, I still pinch myself in disbelief that I was advised by THE legend, the great Marvalee Wake. How does one even process such a thing?! She is, was, and forever will be one of the greatest minds in herpetology, comparative and functional morphology, and evolutionary biology. I'll never forget every word of wisdom she bestowed upon me. Ian Wang has also undoubtedly been one of the most instrumental professors to my academic trajectory. Without hesitation, he let me just show up and functionally join his lab group, wherein I've easily had some of the most intellectually insightful discussions of my entire time at Berkeley. From my first year onward-, he supported me as if I was one of his own graduate students and has unbelievably generous with his time in counseling me about post-graduation career development. I'm further indebted to him for fostering an environment that brought together like-minded folks who are now among my most cherished life-long friends. I very purposely verbally procrastinated during this section in anticipation of how to describe my gratitude toward Eileen Lacey. Immediately upon arrival at Berkeley, she became my champion, my most-trusted faculty confidant, and someone that I unequivocally

consider family. Eileen encouraged me early-on to become a mentor for under-represented minority undergraduates through the Biological Scholars Program: one of the most rewarding experiences of my graduate school journey. She also grows some mean vegetables and is always keen to send you home with pounds of produce after a visit to her house. Her support of the undergraduate and graduate student body is truly unmatched and I'm eternally grateful to have her as an incomparable light in my life.

I could not have gotten through grad school without the amazing cast and crew from the MVZ. Carol Spencer was invaluable in her willingness to drop everything and fish out hundreds upon hundreds (per-week) of tissues in support of my endless sequencing bouts. Michelle Koo was also incredibly supportive of my interest in all things spatial, not to mention the friendliest person one could ever meet. Lydia Smith was and continues to be one of the most impactful people to the success of my research program at Berkeley. She went above and beyond to coach me through molecular methods and is also one of my most cherished friends from school. These three women legitimately deserve an academic holiday named in their honor. Scores upon scores of graduate students wouldn't have a prayer without them.

I'm incredibly thankful to the friends and fellow grad students who have "walked the path" with me while at Cal. Firstly, I owe a giant shout out to my lab mates in the McGuire group. We've been through it all together and I'm very lucky to have them as friends. Dan Portik, Luke Bloch, Sean Reilly, Skip Skipwith, Sarah Hykin, Charlotte Jennings, and Alexander Stubbs were my partners in crime from day one. I couldn't even believe this group of amazing people existed when I arrived for my interview weekend in 2015, and unequivocally I owe my survival and navigation of those first couple of years at Berkeley to them. Sina Amini and Isaac Krone are two of the coolest, funniest people one could ever meet. I'm heartened that they'll be carrying the torch for future McGuire Lab greatness. I basically owe Bryan Bach everything, forever… There's something to be said for the person who seemingly competes with you for the world record of "Most Time Spent Doing Lab Work". I came to Berkeley as a behavioral ecologist having never held a pipette until Bryan guided me through the ins and outs of sequencing methods. Ben Karin and Kelsey Crutchfield-Peters have been my rock for the last few years, both academically and personally (Team BEEF!). A special mention must be made for Ashley Smiley who has been one of my closest friends throughout grad school. Not only did we enter the program together, but she was with me every step of the way as we both feverishly wrote our dissertations together all summer long. I unequivocally could not have done it without her. It's important that also I mention my "Dream Team" of Herpetology students whom I had the honor of teaching during my last semester. I am beyond convinced that this promising group of young scientists will change the world one day and I couldn't be prouder of all their accomplishments. Much love to Amy Wu, Anna Bassias, Ora Younis, Kevin Dang, Ryan Moore, Alex Valencia, Alyssa Perez, and Allisun Wiltshire.

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INTRODUCTION

"There is no other example on the globe of an island so closely surrounded by other islands on every side yet preserving such a marked individuality in its forms of life."

-Alfred Russel Wallace (1880) on the biota of Sulawesi

Earth is concurrently experiencing an alarming rate of global surface temperature change and biodiversity loss. The gravity of a loss in biodiversity is underscored by the elimination of crucial ecosystem services that drive the livable stasis of the planet by its organisms. The crux, is that the most threatened terrestrial ecosystems are also the most biologically productive, and the most rapidly disappearing – the tropical forests. The most pressing priorities to global conservation and ecosystem service persistence are high-payoff research efforts within the regions of greatest species endemism and richness (Myers, et al., 2000).

One such global "biodiversity hotspot" is the Indonesian island of Sulawesi. This island is a top candidate for the most imperiled biodiversity hotspot (Myers et al., 2000), being a leader of global tropical forest loss (Myers, et al., 2000; Hansen et al., 2013). Indeed, integrative ecosystem-level research approaches aimed at understanding the drivers (and future projections) of diversity in Sulawesi are paramount. At the biogeographic interface between the Asian and Australian realms, Sulawesi clearly represents a point of faunal transition – as this island also is home to a diverse assemblage of animals and plants found nowhere else on earth (Lohman et al., 2011). Beyond an abundance of endemics, Sulawesi also contains an abundance of lowland primary rainforests that grade in ecotone up to high elevation cloud forests and alpine habitats atop 3,000 m volcanic peaks, forming unique biogeographic regions. Sulawesi has a number of features that make it among the most interesting islands from the standpoint of biodiversity. The island's endemism is a product of its large area and unusual shape, its isolation from all other landmasses, and its complex tectonic history. Indeed, Sulawesi is the 11th largest island in the world with an area of 174,600 km^2; however, despite these factors, it has received comparatively little attention in the herpetological literature.

The island has substantial topographical relief, with vast areas over 1000 m above sea level, 20 summits >2500 m, and six summits >3000 m. It likely has never been connected via land bridge to the more proximate Sunda continental shelf to its west, nor with the more distant Sahul Shelf (New Guinea, Australia, and their land-bridge islands) to the east, and thus has been isolated for its entire subaerial history of 20-25 million years (Hall 2013). Further, Sulawesi is an aggregate of paleo-islands and this composite nature has been strongly implicated in the process of in situ species diversification and adaptive radiations (Evans et al., 2003a,b; McGuire et al., 2007; Setiadi et al., 2011; Linkem et al., 2012).

Perhaps the most spectacular vertebrate radiation on Sulawesi involves the so-called 'fanged frogs' of the genus *Limnonectes*. This assemblage of ~40 species ranges in adult size from 2–2000+ grams, with many independent derivations of small, medium, large, and giant forms. Communities of six or more sympatric species partition microhabitats and respectively exhibit remarkable reproductive mode variation.

Several *Limnonectes* have generalized anuran reproduction, with eggs fertilized externally in water, and subsequent aquatic larval development. Others breed terrestrially yet proximate to a stream's edge – guarding small clutches (reduced numbers of eggs) deposited on leaves or branches overhanging water. Some species exhibit male nest guarding behavior and larval transport, whereby small clutches of eggs are deposited on land and upon hatching, tadpoles are piggybacked to shallow seeps, puddles, or pools by their resident guardian. Perhaps most dramatic are those with apparent internal fertilization and intra-oviductal maturation, culminating in the live-birth of tadpoles – a reproductive mode unique among all other frogs on Earth!

The research described herein aims to elucidate the taxonomic uncertainty in the Sulawesi *Limnonectes* assemblage and systematically characterize the morphological, physiological, and life history features associated with the assemblage's astounding phenotypic variation. Through extensive field collecting, focal observations, and assemblage-wide experimentation conducted over six expeditions to Indonesia, this body of work helps to offset the current knowledge gap in terms of how differential reproductive strategies and ecological niche partitioning amalgamate to produce replicate instances of cryptic and / or sympatric speciation events. The analytical approaches employed throughout the following chapters address the need for comprehensive characterizations of the ecological gradients present on Sulawesi in effort to identify both the environmental thresholds and population genomic structure that drive variable spatial patterns of *Limnonectes* species' occurrence and habitat selection propensities across the island. Furthermore, some of the findings herein are completely without precedent because due to the integration of behavioral ecology and eco=physiology components encompassed within the overall study design. These types of studies are sorely lacking across herpetofauna in general, let alone for a rare and unique assemblage such as this.

In Chapter 1, I describe and a new species of terrestrially-nesting fanged frog endemic to South Sulwesi – now the smallest-known species among its Sulawesi congeners. To diagnose the new fanged frog, "*Limnonectes phyllofolia*", I calculate metrics of genetic distance (based on 16S ribosomal RNA), conduct multivariate statistical analyses on a 20-character morphometric dataset, and robustly compare acoustic facets of their reproductive advertisement calls against the other *Limnonectectes* species known from the Southwest peninsula. In Chapter 2, I characterize the discovery of two fascinating cases of elevational speciation between known South Sulawesi lowland species and their respective, heretofore undescribed highland analogs. I preliminarily identify the instances of cryptic speciation by comparing mitochondrial sequences across three genes. I then used two separate Next-Generation Sequencing (NGS) methods to: (1) perform phylogenetic species delimitation using the mitochondrial genomes, and (2) test for population structure without admixture using targeted-capture exonic data from ~6,000 loci across the nuclear genome. I subsequently used statistical comparative morphometrics to diagnose the two novel upland species, "*L. kejutan"* and "*L. diatas*". In Chapter 3, I report findings from eco-physiological water loss experiments that I conducted across the Sulawesi assemblage while in the field. With this study, I identify significant differences in physiological dehydration across eight fanged frog species. Moreover, I discuss unexpected findings regarding apparent behavioral hydro-regulation and demonstrate the importance of deriving species-specific *in situ* metrics for water loss when aiming to produce spatially explicit, mechanistic

biophysical models that accurately reflect differential niche use. Taken together, the sampling efforts and focal observations, molecular analyses, and eco-physiological trial results that are reported herein, constitute novel contributions to what little is currently known to science regarding the taxonomy and natural history of Sulawesi fanged frogs. Hopefully, this work will serve as a springboard for future research that aims to profile the comprehensive suite of ecological and evolutionary mechanisms facilitating lineage divergence in this radiation.

CHAPTER 1

A New Species of Terrestrially-Nesting Fanged Frog (Anura: Dicroglossidae) from Sulawesi Island, Indonesia

ABSTRACT

Herein, we describe a new species of terrestrially-nesting fanged frog from Sulawesi Island, Indonesia. Though male nest attendance and terrestrial egg deposition is known in one other Sulawesi fanged frog (*Limnonectes arathooni)*, the new species exhibits a derived reproductive mode unique to the Sulawesi assemblage; male frogs guard one or more clutches of eggs festooned to leaves or mossy boulders one to two meters above small slow-moving streams, trickles, or seeps. This island endemic has thus far been collected at three sites on Sulawesi: one in the Central Core of the island, and two on the Southwest Peninsula – south of the Tempe Depression (a major biogeographical boundary). The new *Limnonectes* has the smallest adult body size among its Sulawesi congeners – with a maximum snout-vent length of about 30 millimeters. Beyond its unique reproductive behavior and body size, the species is further diagnosed on the basis of advertisement call and genetic distance from sympatric fanged frogs. The discovery and description of the new species highlights the remarkable reproductive trait diversity that characterizes the Sulawesi fanged frog assemblage despite that most species in this radiation have yet to be formally described.

INTRODUCTION

The Asian Dicroglossid fanged frogs (genus: *Limnonectes*) include over 70 species and are stunningly complex in their reproductive biology. For example, two Malay species, *L. hascheanus* and *L. limborgi* exhibit terrestrial egg guarding by males in conjunction with nidicolous larval ontogeny: larvae hatch as free-living tadpoles yet remain in a nest guarded by the male, surviving solely on nutrients from the yolk sack (Inger and Stuart 2010; Rowley and Altig 2012**)**. Four species of *Limnonectes* from Borneo, *L. kuhlii*, *L. blythii*, *L. ibanorum*, and *L. ingeri* are "voiceless", lacking a vocal sack for advertisement calling (Emerson 1992). Among them, the breeding biology of *L. blythii* includes female biased sex ratios, and males that both guard and defend limited shallow oviposition sites on gravel bars along fast-moving streams. Females of this species patrol the available nest sites to choose from the suite of deposition locations and resident male guardians (Emerson 1992). In Borneo and the Philippines, both male and female *L. palavanensis* vocalize to some degree (Vallejos et al. 2018). Terrestrial egg deposition is exhibited by both *L. palavanensis* and *L. parvus*; however, upon hatching, larvae are subsequently transported on the back of the male to isolated water basins in the forest (Vallejos et al. 2018).

The radiation of fanged frogs on the Indonesian island of Sulawesi likewise features remarkable variation in breeding biology and reproductive modes (Brown and Iskandar 2000; Evans et al. 2003a; Iskandar et al. 2014; Setiadi et al. 2011). This poorly studied assemblage likely includes 15–20 species, only five of which have been formally described in the literature: *L. heinrichi* (Ahl 1933), *L. modestus* (Boulenger 1882), *L. arathooni* (Smith 1927), *L.*

microtympanum (Van Kampen 1907), and *L. larvaepartus* (Iskandar et al. 2014). Recent field investigations suggest that at least one species (referred to as *L.* "*Sp. I*" in Evans et al. 2003a; Setiadi et al. 2011) is voiceless, as it lacks vocal sacs and buccal slits. Brown and Iskandar (2000) described terrestrial egg deposition in *L. arathooni* from Sulawesi, concurrent with other interesting observations of tadpoles spontaneously emerging from their egg capsules when nests were disturbed. Males of this species often guard multiple nests deposited on steep stream banks until newly emerged larvae wriggle down to the stream below. The reproductive modes of the Sulawesi assemblage are so varied, in fact, that they can be used as primary characters for species diagnoses. In perhaps the most striking example, the reproductive biology of the recently described *L. larvaepartus* includes internal fertilization, intraoviductal larval maturation, and the birth of free-swimming tadpoles – a first among all anurans known to science (Iskandar et al. 2014; Kusrini et al. 2015).

Here, we report a new species of *Limnonectes* from Sulawesi, and only the second species from the island found to exhibit terrestrial egg deposition and male egg guarding. *Limnonectes* diversity on Sulawesi is poorly understood in part because of the difficulty in discriminating between morphologically and phenotypically similar animals, as well as the challenge of recognizing when morphological variation reflects interspecific differences versus intraspecific polymorphism. Our description exemplifies this challenge in that the new species occurs in sympatry with *L. arathooni*, the only other Sulawesi species documented to deposit eggs on land. Moreover, the two species are somewhat similar in size and appearance. We show herein that the new species can be diagnosed on the basis of body size, advertisement call, egg deposition behavior, and genetic distance. Unlike *L. arathooni* that deposits eggs in either streamside leaf litter or in holes in stream banks, the new species deposits nests 1–2 m off the ground on leaves or mossy boulders. These "leaf nests" overhang small, slow-moving forest streams, puddles, or seeps. As with other fanged frogs in which males exhibit parental care, we observed that males of this novel species attend one or two nests until tadpoles emerge and drop into the water below (see Figure 1).

METHODS

Field sampling.—Field work and animal collections were undertaken with both research and export permits in collaboration with the Indonesian Institute of Sciences (LIPI), and were granted by the Indonesian Ministry of Research, Technology, and Higher Education (RISTEK). Prior to conducting this research, animal handling and specimen preparation protocols were approved by the UC Berkeley Institutional Animal Care and Use Committee (Protocol : R279). Herpetological surveys on Sulawesi were conducted on Gunung Lompobatang in 2005, Gunung Balease in 2010, and Gunung Bontosiri (Bantimurung National Park) in 2014 (Figure 2). Hand-captured specimens were fixed using 10% buffered formalin. The sex of each frog was determined either by viewing advertisement call behavior prior to capture, or by gonadal inspection during specimen preparation. Fixed specimens were subsequently stored in 70% ethanol and deposited at the Museum Zoologicum Bogoriense (MZB) in Cibinong, Indonesia, or the Museum of Vertebrate Zoology (MVZ) at UC Berkeley.

Morphology.—Morphological measurements were taken using digital calipers (to nearest 0.01 mm) including: head length (HL); head width (HW); snout-vent length (SVL); tibia length (TL);

interorbital distance (IO); eye diameter (ED); internarial distance (ID); eye-nostril distance (EN); foot length (FL); tympanum diameter (TD); thigh length (THL); snout length (SL); hand length (HAL); forearm length (FLL); eye-tympanum distance (ETD), snout-nostril length (NS); upper arm length (UAL); lower arm length (LAL); and body width (BW) following Watters et al. (2016). We also measured the length of the odontoid process (OPL) – the distance between the lower margin of the mandible and the top of the fang-like process protruding upward from the mandible. Notations and terminology for the digital webbing formula and relative finger lengths followed Guayasamin et al. (2006) and Stuart et al. (2020). In brief, fingers and toes were indicated by roman numerals, while Arabic numerals were used to indicate the position of the webbing on each phalange relative to the positions of the toe disc, intercalary cartilage, and subarticular tubercles. To validate our presumption that the new species differs morphologically from its sympatric congener (*L. arathooni*), we performed significance tests using a multivariate analysis of variance (MANOVA) on our morphological measurements. To account for individual- and locality-based body size variation, we first performed a principal components analysis (PCA) on the measurements taken for both species. We recorded the percent variance attributed to each PC by the morphological characters for downstream interpretation of significance testing and extracted the PC scores from each of the principal components to use as variables in the MANOVA.

Acoustic Sampling.—We conducted acoustic surveys in the field, recording the advertisement call of male *Limnonectes* at a distance of 0.5–1.5 m using either a handheld solid state recorder (Marantz Professional, USA: PMD661MKII) and a stereo shotgun condenser microphone (Sennheiser: MKH 60-P48), or an iPhone attached to an external microphone. The resultant waveform audio format files were analyzed using Raven Pro Sound Analysis Software – version 1.5 (Bioacoustics Research Program, 2014). For each recording, we viewed both waveform and spectrograms that were calculated using a Fast Fourier Transformation size of 512. We analyzed calls individually, thus, if recordings contained more than one call per animal, each call within the recording was isolated prior to the analysis. This demarcation resulted in a dataset consisting of 16 *L. phyllofolia* calls across 3 individuals (JAM 14390, JAM 14393, and JAM 14394) and 10 *L. arathooni* calls across five individuals (JAM 14428, JAM 14914, JAM 14946, JAM 15066, and one non-vouchered animal). For each call, we measured number of notes, mean dominant frequency (Hz), call duration (in seconds), and pulse rate (in notes per second). We then performed cluster analysis on the call characters for both species in R – Version 3.5.1 (R Core Team, 2018). To account for any potential statistical non-independence, multicollinearity, autocorrelation, and pseudoreplication in the data, we performed a PCA on the aforementioned call characters. We recorded the percent variance attributed to each PC by the call characters for downstream interpretation of significance tests and extracted the PC scores from the four principal components. We then used the PC scores as variables in a MANOVA to test for significant differences between the call characters of the two species.

Genetic Sampling.—Field-collected liver tissue samples from *L. arathooni* (n = 20), *L. microtympanum* (n = 46), and (n = 35) individuals of the new species were preserved in RNA Later and subsequently salt extracted to obtain genomic DNA. We used the polymerase chain reaction (PCR) to amplify a 515–530 base pair (bp) fragment of the 16S rRNA marker with primers 16S-H3062 (5'-CCGGTTTGAACTCAGATCA-3') and 16SB-FROG (5'-CGCCTGTTACCAAAAACAT-3'). PCR conditions were as follows: denaturation at 94°C – 2 min, 35 cycles (denaturation at 94°C – 45 s, annealing at 53°C – 30 s, extension at 72°C – 1

min), and final extension at 72°C for 1 min. Resultant amplicons were purified with ExoSAP-it (Applied Biosystems) and cycle sequenced with our amplifying primers using BigDye v 3.1. We then used ethanol (125mM EDTA) precipitation to purify the cycle sequence products and ran the samples on an ABI 3730 automated DNA sequencer (Applied Biosystems). We edited and manually aligned all sequences in Geneious 9.1.8 (Biomatters). We then calculated uncorrected patristic distances between samples using sequences for *L. microtympanum*, *L. arathooni*, and the new species in PAUP 4.0a build 168 (Swofford, 2002).

Nomenclature acts.—The published edition of this article conforms to the International Code of Zoological Nomenclature (ICZN). The nomenclature acts herein are registered within the ICZN system on Zoobank.org. The Zoobank Life Science Identifier (LSID) for this published work and its nomenclature acts can be viewed online by visiting https://www.Zoobank.org, and referencing: urn:lsid:zoobank.org:act:E9A254BE-026A-42A0-8A33-8CDD6D6C7715. This species was referenced in Smith (1927) under the name *Rana palavanensis*, and as *Rana microdisca leytensis* in the British Museum of Natural History Catalogue.

RESULTS

Limnonectes ***phyllofolia*** sp. nov.

(Figures 2–4)

Etymology.—We have informally referred to this species as *Limnonectes* sp. "leaf-nester" in reference to its characteristic reproductive mode. We therefore opted to memorialize this in its formal specific epithet, "*phyllofolia*", which is derived from the combination of the greek *fýllo* – meaning "leaf", and *foliá* – meaning "nest".

Holotype.—An adult male (JAM 14394), collected 25 June 2014 at 22:08 h, from Sulawesi Island, Indonesia: Sulawesi Selatan Province: Kabupatan Maros: Kecematan Mallawa: Desa Bontosiri: Bantimurung National Park (S 04.81668, E 119.84586 ± 5 m) at 592 m by J. A. McGuire and Djoko T. Iskandar.

Paratypes.— JAM 11313–4, 11322–7, 11329, 11330, 11333–4, seven adult males, one adult female and four juveniles, collected by J. A. McGuire, S. B. Reilly, A. L. Stubbs, and G. Ramadhan on 19 October 2010; JAM 11345, one adult male, collected by J. A. McGuire, S. B. Reilly, A. L. Stubbs, and G. Ramadhan on 21 October 2010; JAM 11397–11402, six adult males, collected by J. A. McGuire, S. B. Reilly, A. L. Stubbs, and G. Ramadhan on 24 October 2010; all from 692 m elevation on Gunung Balease (S 02.50884, E 120.47936 ± 6 m). JAM 11432, 11440, 11442, four adult males and one juvenile, collected by J. A. McGuire, S. B. Reilly, A. L. Stubbs, and G. Ramadhan on 26 October 2010; all from 760 m on Gunung Balease (S 02.50579, E 120.48181 ± 7 m). JAM 14371–2, 14378, 14380, 14382, 14384, 14391, seven adult males and one adult female, all with the same data as the holotype. JAM 14325 and 14327, two adult males; collected by J. A. McGuire and D. T. Iskandar on 24 June 2014 at the type locality.

Distribution.—*Limnonectes phyllofolia* is a Sulawesi endemic, known only from the three collection localities (Figure 2) described herein (Desa Bontomaranu on Gunung Lompobatang,

Gunung Balease, and Desa Bontosiri in Bantimurung National Park). Bantimurung National Park and Desa Bontomaranu are on the Southwest Peninsula of Sulawesi south of the low-lying Tempe depression, an important biogeographical boundary for many taxa including tarsiers, macaques, toads, and other fanged frog congeners (Evans et al. 2003a,b; Groves and Shekelle 2010). The third locality, Gunung Balease, is located in the southeastern quadrant of Sulawesi's Central Core, thereby demonstrating that the range of *L. phyllofolia* spans the Tempe Depression biogeographical boundary. The three collecting localities range from a low of 495 m elevation at Bontosiri to 1173 m at Bontomaranu. We expect that this species occurs broadly across the Southwest Peninsula in the lowlands up to perhaps 1200-1300 m in elevation wherever there is sufficiently intact habitat, while noting that there is desperately little intact habitat in this elevational range outside of Bantimurung National Park. In 2016, we sampled extensively on Gunung Bawakaraeng on the Southwest Peninsula between 1520 m elevation and 2800 m in mildly disturbed-to-pristine habitats and did not detect this species, suggesting that it is absent from higher elevation mossy forest. The extent of this species' range in the Central Core is much more difficult to predict. It is possible the species is broadly distributed in intact low to mid-elevation habitats, but pristine forest is rare in the lowlands of the Central Core and where we have surveyed such habitats (e.g., within Lore Lindu National Park), we did not encounter this species. It is possible that this species was once widespread in lowland forests of the Central Core but is now range-restricted because of loss of habitats at lower elevations.

Diagnosis.—We have found *Limnonectes phyllofolia* living in sympatry/syntopy with the described species *L. arathooni* (at Bontomaranu) and *L. microtympanum* (at Bontomaranu and Bontosiri). With regard to the informally recognized undescribed species of Evans et al. (2003a, b) and Setiadi et al. (2011), we have found *L. phyllofolia* in sympatry with the undescribed *L.* "*sp. 2*" (at Gunung Balease), with the undescribed *L.* "*sp. T*" (at Gunung Balease), and with the undescribed *L.* "*sp. J*" (at Gunung Balease). The range of *L. phyllofolia* likely also overlaps with the ranges of *L.* "*sp. D*", *L. "sp. G2*", and *L.* "*sp. I*". The known geographic distribution of *L. phyllofolia* does not overlap with the ranges of *L. modestus*, *L. heinrichi*, "*L. sp. 1*", or *L.* "*sp. J2*". Regardless of whether there is range overlap, *L. phyllofolia* is highly genetically distinct from each of these congeners.

Limnonectes phyllofolia is distinguished morphologically from all other described Sulawesi *Limnonectes* by the following combination of characters: small adult size (a maximum SVL of ~30 mm, Table 1), highly reduced webbing, and the presence of a post-orbital skin groove which appears as an off-white stripe on lighter-colored individuals (though reduced webbing and the postorbital groove have also been reported in *L. arathooni* (Iskandar et al. 2014). *Limnonectes phyllofolia* can also apparently be distinguished from all other Sulawesi *Limnonectes* on the basis of its reproductive behavior (though we admittedly still don't know the reproductive modes of several species). Namely, this species is terrestrially-nesting, depositing its masses in tightly packed clutches of 10-20 eggs on leaves or boulders festooned with a thick layer of wet moss immediately over- or adjacent to small streams and seeps. Though nest attendance is also known in *L. arathooni*, an only slightly larger congener; nest sites of *L. arathooni* occur on steep stream banks, and larvae hatch from eggs when disturbed, sliding or wriggling down the bank into water (Brown and Iskandar 2000). In contrast, sites of *L. phyllofolia* occur ~1–2 m off the ground, either on leaves of ferns, saplings, or other plants that overhang small slow-moving streams, seeps, or puddles, or on elevated boulders overhanging water (Figures 1, 4). It is unclear whether the new species overlaps in range with the recently

described *L. larvaepartus*, a small species that is nevertheless substantially larger in adult body size, with mean male and female SVL of *L. larvaepartus* being reported as 37.4 mm and 40.2 mm, respectively (Iskandar et al. 2014). As *L. larvaepartus* demonstrates a reproductive mode unique to all frogs (internal fertilization with live birth of tadpoles), the new terrestrially-laying species can be easily distinguished from its congener by this criterion (Iskandar et al 2014). Though morphological measurements suggest that *L. arathooni* can be distinguished from *L. phyllofolia* by their larger size (*L. arathooni* average SVL = 35.23 mm, range: 29.47–44.3 mm; *L. phyllofolia* average SVL = 27.03 mm, range: 21.53–30.13; Table 2), field identification between of the two species may be difficult from size alone. Thus, *L. phyllofolia* can also be distinguished from *L. arathooni* and all other *Limnonectes* on the basis of call: a rapid series of clicks unlike any of its Sulawesi congeners.

Description of holotype.—Adult male, SVL 28.12 mm, head large and wide relative to body size: HL 9.4 mm, HW 11.38, 33% and 40% of SVL, respectively; head slightly wider than long, 115% of HL, and wide relative to the body (HW/BW = 116%); internarial distance (2.58 mm) roughly equal to the distance from the nostril to the anterior margin of the eye (though in paratypes the latter may be slightly longer (mean IN/EN = 102%)); eye large, 4.14 mm in diameter, 15% of SVL, and 44% of HL; interorbital distance 2.98 mm, slightly convex, and 11 % of SVL; tympanum round, 2.56 mm in diameter, 9% of SVL; odontoid processes (fang-like boney projections protruding upward from the mandible) small, 1.14 mm.

There is a distinct supra-tympanal fold of skin initiating at the center posterior margin of the eye that extends over and around the tympanum, terminating just above the arm.

Arms stocky and short, with forearm length (FAL = 5.25 mm) roughly equal to upper arm length (UAL = 5.26 mm), and both distances 64.4% and 64.5% of hand length (HL = 8.15 mm), respectively; fingers long, with hand length 29% of SVL and 86% the length of the head; relative finger lengths **III** > **I** > **II** > **IV** with webbing completely absent from fingers (Figure 5); body squat, a third wide as it is long (BW/SVL = 34%); hindlimbs long and slender, with tibia length roughly equal to thigh length (TL / THL = 98.8%), both distances only slightly longer than the foot (FL = 15.48 mm); hindfoot webbing highly reduced (relative to sympatric *Limnonectes* spp.) with webbing formula **I** $1^{1/2} – 2^{-}$ **II** 1 – 1 **III** $3^{-} – 1$ **IV** $2^{-} – 3$ **V** (Figure 5); skin fairly rugose, especially behind the eyes, but also dorsally and laterally along the flanks; skin smooth on the ventral side of the limbs and trunk.

Coloration.—Prominent markings (even in preservative) include: a small (< 1 mm), perfectly circular light-colored spot in the center of the snout, a light-colored interorbital bar, a light-colored delta-shaped snout patch initiating between the eyes and terminating at the snout, and vertical white lip bars alternating across the length of the mandible and terminating beneath the tympanum, eyes, nares, and snout, respectively. In individuals without a light-colored snout patch, the interorbital bar is dark and may also present with a distinct groove in the dermis (Figure 3). Dark leg bars are usually prominent, especially on the thigh, and appear to run continuously across the thigh, tibia, and tarsus when the legs are not extended. Often, the leg bars will be faded or absent across the tibia. In life, *L. phyllofolia* coloration ranges from very dark, to light cinnamon brown (Figure 2). Two color morphs are generally exhibited: standard, and barred. Standard morphs are dorsally brown, usually grading to lighter brown or cream-colored flanks (e.g., Figs. 1B, D; 3A [JAM14382, 14378, 14387]). Barred individuals have two light-colored stripes that initiate postorbitally and run the length of the dorsum on either side of the

spine and urostyle, terminating at the vent (e.g., Figures 1A, C, E; 3A [JAM14395]). Gray-brown mottling is prominent on the cream-colored venter, initiating at the snout and extending down the trunk. The same mottling is also present around the ventral margins of the legs, where the cream-colored background may in some cases grade to a pale yellow color.

Eggs and tadpoles.—Average *Limnonectes phyllofolia sp. nov.* clutch size of $n = 9$ nests collected in concert with adult male specimens was 15 (range = 10–21 eggs) (Figure 4). The roughly 5 mm diameter eggs are sturdy, appear perfectly spherical, and are tightly packed within the mound-like nest. Embryos are surrounded by clear jelly, such that developing larvae are easily visible. In the field, we observed that both late-stage and non-viable eggs become cloudy, and are likely susceptible to mold and other fungi when not accompanied by a guardian male.

Natural history.—Of the three known localities where *Limnonectes phyllofolia* has been found, one was in pristine karst forest habitat within Bantimurung National Park, one was in mature regenerated forest adjacent to primary forest on Gunung Balease, and one was adjacent to a large, high-flow waterfall in a heavily disturbed village setting (Desa Bontomaranu on Gunung Lompobatang). Despite having observed these frogs in habitats of variable quality, the fact that we have not found this species at the dozens of additional (mostly disturbed) sites that we have surveyed throughout the Southwest Peninsula and southern Central Core suggests to us that this species is likely almost entirely restricted to mature natural forest habitats below ~1100 meters in elevation. It is quite possible that this lowland obligate, and the sampling site at the base of G. Lompobatang (1100 m elevation) may itself be an elevational outlier. Mature lowland forest is sufficiently scarce on Sulawesi to the extent that any species restricted to these habitats will be found primarily in protected areas such as Bantimurung National Park, or in remote sites away from human habitations that retain some suitably natural forest vegetation (e.g., our 700–900 m elevation collecting sites on Gunung Balease).

Limnonectes phyllofolia is one of several small Sulawesi fanged frog species that are fairly terrestrial in their habits. Although reproduction is associated with small streams and seeps, individuals can be found far from any free-flowing water in open forest leaf-litter. We collected several individuals on Gunung Balease that were hundreds of meters from the one breeding site that we identified. That breeding site was a spring-fed seep that emerged from the forest floor, flowed slowly for perhaps 50 meters down slope, and then disappeared. We were not aware of a more continuous stream within 1.5 km of this spring-fed seepage system. Small discontinuous aquatic habitats are likely to be colonized by smaller more terrestrial *Limnonectes* species such as *L. phyllofolia*, and these habitats are unlikely to be utilized by larger, more stream-adapted *Limnonectes* species that might be both predators on-, and competitors with this species.

At both Gunung Balease and Gunung Bontosiri, we found many males perched on egg clutches immediately over small, slow-moving streams. At Bontosiri, we also found a small number of males on clutches adjacent to a moderately large stream (4–5 m in width) with a faster flow regime, as well as on the margin of a large pool (12 m x 6 m) nearly devoid of flow. The egg clutches were either on the green leaves of tree saplings or ensconced in moss on boulders. We only saw males attending egg clutches at night and unattended egg clutches were observed on Gunung Balease during the day. Based on the close proximity of some clutches (6–12 inches apart), it appeared that some individual males might be guarding two clutches at once, although we never confirmed this through observation of a male switching between clutches. Although we

observed many males on egg clutches, none were vocalizing. Three males were observed calling and none were on or near egg clutches. Having not witnessed amplexus, it remains unclear which sex chooses the site for egg deposition, but it seems possible that females choose males in part based on their territory quality. It is also possible, however, that the females decide where the clutches will be deposited once engaged in amplexus.

Variation.—In addition to variations in coloration described above, this species also varies substantially in degree of rugosity of the dorsal skin surfaces, with some individuals quite rough in appearance and other smooth. Morphometric variation among the paratypes and holotype are described in Table 1.

Morphometric comparisons.—Though *L. phyllofolia* is objectively smaller than *L. arathooni*, it remains possible that observers might confuse the two species in the field. Herein, we report statistical support for the new species being distinguishable from its sympatric congener based upon body size and limb lengths (Figure 6). Eigenvalues from the cluster analysis on morphological characters revealed that the first three principal components (PCs) accounted for 89.4% of the total variance across the morphological dataset. Important contributors of variance to the first three PCs were: (1) THL, LAL, HW, SVL, TL, FL, HAL, FLL, BW, and UAL for PC-1, (2) ED, TD, SN, ETD, and SL for PC-2, and (3) TD, ED, SN, OPL, EN, and ETD for PC-3. MANOVA results on the PC scores were highly significant, highlighting the substantial differences between the morphological characters of the two species ($\alpha < 0.001$; $P = 9.38e^{-07}$). The above ten characters in PC-1 explained most of the total variance (82.47%) in the model and PCA scores in this dimension differed significantly ($\alpha < 0.001$; $P = 5.408e^{-09}$).

Advertisement call.—Here, we compare the advertisement call of *L. phyllofolia* with its most similar Sulawesi congener, *L. arathooni* – a species with which it is also sympatric on Sulawesi's Southwest Peninsula. Indeed, *L. phyllofolia* can be easily distinguished from *L. arathooni* (and all other Sulawesi congeners) on the basis of call – a rapid series of clicks quite unlike the high-pitched chirps uttered by *L. arathooni*. Examples of calls from both species are shown in FIGURE 7. Call duration of *L. phyllofolia* ranged from 0.596–2.86 seconds (sec) ($\overline{x}$ = 1.3 sec, SD = 0.86), while the range of call duration recorded for *L. arathooni* was 0.355–3.537 sec ($\overline{x}$ = 1.69 sec, SD = 1.19). Number of notes from calls of *L. phyllofolia* ranged from 11–54 notes ($\overline{x}$ = 32.25 notes, SD = 16.38), while *L. arathooni* calls were composed of substantially fewer notes (range = 2–7 notes, $\overline{x}$ = 3.4 notes, SD = 1.71). The pulse rate of calls collected from *L. phyllofolia* varied little, ranging from 16.78–19.44 notes per second (NPS) ($\overline{x}$ = 18.34 NPS, SD = 0.62), while the pulse rate of calls from *L. arathooni* ranged from 0.57–6.13 NPS ($\overline{x}$ = 3.16 NPS, SD = 2.54). Dominant frequency of *L. phyllofolia* ranged from 1142.70–2818.89 Hz ($\overline{x}$ = 2046.89 Hz, SD = 536.81), while dominant frequency of across *L. arathooni* calls ranged from 1751.40–2813.67 Hz ($\overline{x}$ = 2374.55 Hz, SD = 312.09).

Cluster analysis with PCA resulted in the first three of four PCs accounting for 98.38% of the total variance across the call character dataset (Figure 8A). Important contributors of variance to the first three PCs were note number and pulse rate for PC1, dominant frequency for PC-2, and call duration and dominant frequency for PC-3 (Figure 8B). MANOVA results on the PC scores were highly significant, highlighting substantial differences between call characters of the two species ($\alpha < 0.001$; $P = 2.383e^{-16}$). For PC-1, which was explained by the variances of pulse rate and note number, score differences were significant between the two species at $\alpha < 0.001$ ($P = 4.973e^{-07}$). PC-2 was explained mostly by the variance contributed by dominant frequency, and

scores again differed significantly between the two species at $\alpha = 0.001$ ($P = 9.592e^{-03}$). There were no significant differences between PC scores of PC-3 and PC-4. Call differences between *L. phyllofolia* and *L. arathooni* are quite clear, both audibly and via visual inspection of sonograms (Figure 7), though here we offer statistical validation that *L. phyllofolia* can be diagnosed from its similarly-sized sympatric congener on the basis of note number, pulse rate, and dominant frequency. The resulting biplot from the PCA (Figure 8A) shows clustering in the data by species as well as the variance contribution by call characters for the first three PCs.

Genetic distance.—Using data collected for another project, we calculated uncorrected patristic distances for a 426 bp fragment of the 16S mitochondrial gene for *L. arathooni*, *L. microtympanum*, and *L. phyllofolia*. We found that the currently recognized species *L. arathooni* and *L. microtympanum* exhibited a minimum uncorrected pairwise genetic distance of 3.85% from one another, whereas these species exhibited 8.22% and 8.23% genetic distances from *L. phyllofolia*, respectively.

DISCUSSION

The poorly studied radiation of Sulawesi fanged frogs likely represents the most diverse amphibian assemblage on the island. Five species are formally described, yet recent studies, ongoing field surveys, and genetic analyses indicate that the assemblage is comprised of at least 15 species (Evans et al. 2003a; Setiadi et al. 2011; Iskandar et al. 2014). Moreover, this group has been suggested to represent an adaptive radiation (Setiadi et al. 2011). For example, Sulawesi *Limnonectes* exhibit at least 450-fold variation in adult body mass, important differences in microhabitat use and associated eco-morphological phenotypes, a diverse array of reproductive modes, and a large number of species that can be found in sympatry (we have found at least 6 species co-occurring in the Central Core and on the Southeast Peninsula, and the known extents of species geographic ranges leave possible that as many as 11 species might be found together at select localities in the eastern Central Core). A suite of sympatric ecomorphs can reliably be found in lowland and montane habitats across the island. For instance, several large and moderately-large fully-webbed species (*L. heinrichi, L. microtympanum*, *L.* "sp. D", *L.* "sp. I", *L.* "sp. 2") are strongly associated with large, fast moving streams. Smaller extensively-webbed species (such as *L. modestus* and *L. larvaepartus* are sometimes found on large streams but are more typically associated with smaller streams and seeps. More terrestrial leaf-litter specialists such as *L. arathooni*, *L.* "sp. G2", *L.* "sp. J", *L. "*sp. J2", *L.* "sp. T", and *L.* "sp. 1" have much reduced webbing even though most utilize small streams for reproduction. *Limnonectes phyllofolia* is an example of this latter terrestrial ecomorph: it is the smallest known Sulawesi fanged frog, it has greatly reduced interdigital webbing, and we have collected the species quite far from any source of surface-water. This species, like the other more terrestrial species, appears to avoid larger streams and rivers, instead breeding adjacent to small streams and seeps where it exhibits a reproductive mode wherein eggs are deposited on leaves or in thick, moist moss overhanging water. There remains much to be done in the way of identifying and characterizing ecomorphological variation in Sulawesi fanged frogs, but the discovery and description of *L. phyllofolia* provides further evidence that *Limnonectes* frogs partition habitats and niches on the island of Sulawesi.

At this time, there is still considerable uncertainty regarding the full geographic distribution of *L. phyllofolia*, particularly regarding its range within Sulawesi's Central Core. We have failed to find additional populations despite having conducted extensive fieldwork in the southern half of the Central Core where *L. phyllofolia* should be expected. However, the Central Core is primarily composed of an upland plateau and it's possible that this species may be (or may have once been) restricted to the lower elevation margins of the main massif with anthropogenic habitat modification resulting in extirpation of the species over much of its range north of the Tempe Depression. Further survey work is clearly required if we are to the geographic extent of this species range, and it is essential to locate and investigate lowland regions in the Central Core that retain intact forest habitats.

Indonesia has been repeatedly identified as a global biodiversity hotspot (Iskandar et al. 1996; Mittermeier et al. 1999; Myers et al. 2000), and Sulawesi is itself a global hotspot of biodiversity and endemism, and a high-priority, imperiled region of conservation concern (Iskandar and Tjan 1996; Lohman et al. 2011; Meyers et al. 2000; Whitten and Henderson 2002). Nevertheless, the herpetofauna of this island remains poorly documented with many of the currently recognized species actually representing species complexes, and numerous additional morphologically distinct species awaiting taxonomic description (see Koch 2011). Here, we add an additional species to the known roster of anurans inhabiting Sulawesi, while noting that much work remains to fully characterize the herpetofauna of this remarkable island.

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FIGURES AND TABLES

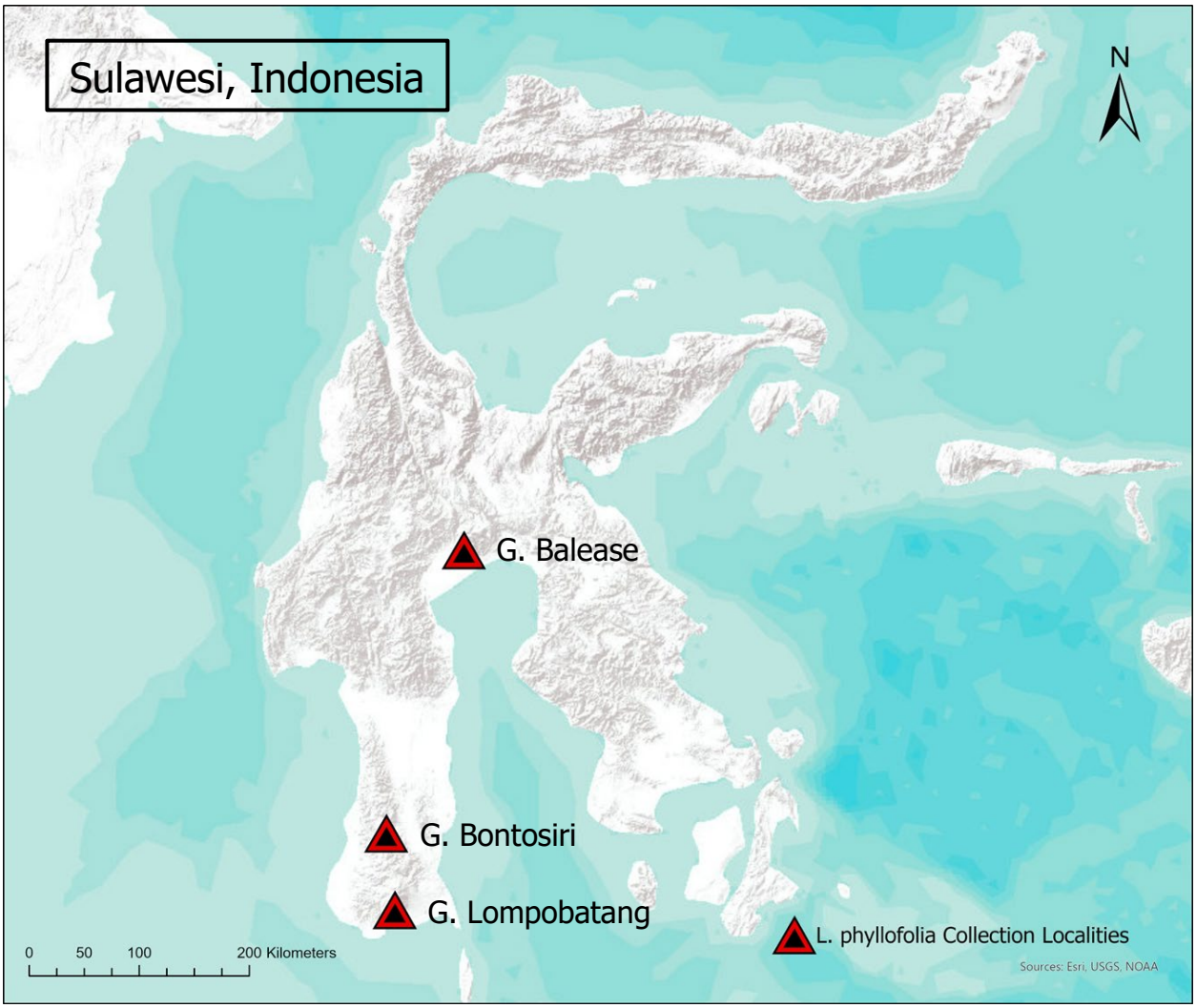

FIGURE 1—Collection localities of *Limnonectes phyllofolia* sp. nov. on Sulawesi Island, Indonesia. The Gunung Lompobatang collection site was located on the Southwest Peninsula near the town of Cikoro. The Gunung Bontosiri (type locality) collection site was also located on the Southwesi Peninsula within Bantimurung National Park. The third collection site at Gunung Balease was located in Sulawesi's southeast Central Core near Sukamaju District.

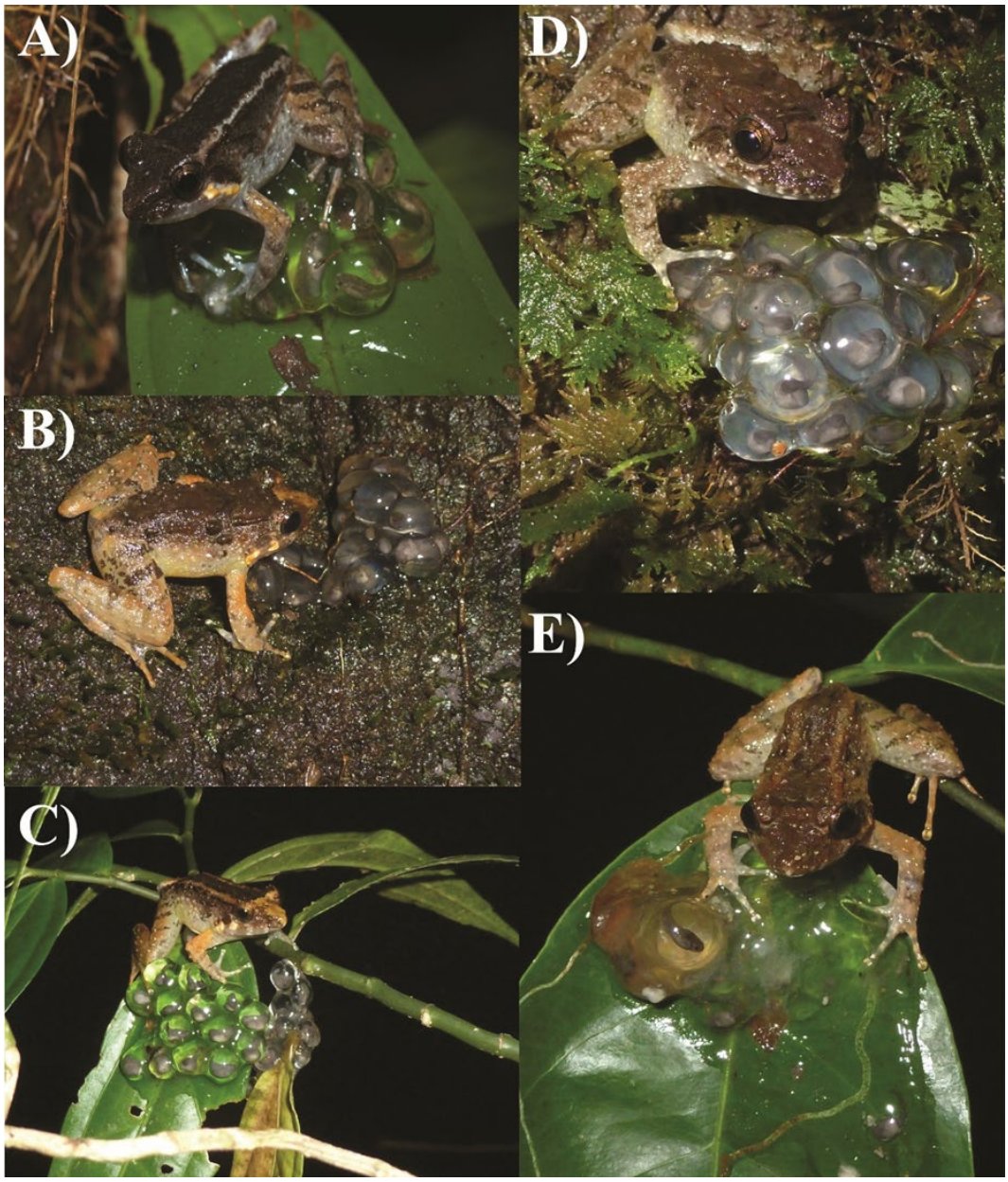

FIGURE 2—Images of *Limnonectes phyllofolia* sp. nov. in life. (A) A male *L. phyllofolia* (no voucher) guards an egg clutch on a leaf 1 meter above a seep in Bantimurung National Park. (B) A male *L. phyllofolia*, JAM14373, guards an egg clutch 0.6 m up on a 2 m tall mossy boulder overhanging a stream in Bantimurung National Park – 25 June 2014. (C) A male *L. phyllofolia*, JAM14396, guards an egg clutch on a leaf 0.2 m above a puddle in Bantimurung National Park – 25 June 2014, 22:25 h. (D) A male *L. phyllofolia*, JAM14387, guards an egg clutch on a mossy boulder 1 m above a 1 m wide cascading stream in Bantimurung National Park – 25 June 2014, 21:38 h. (E) A male *L. phyllofolia* (no voucher) guards an egg clutch on a leaf while larvae hatch and drop into the water below.

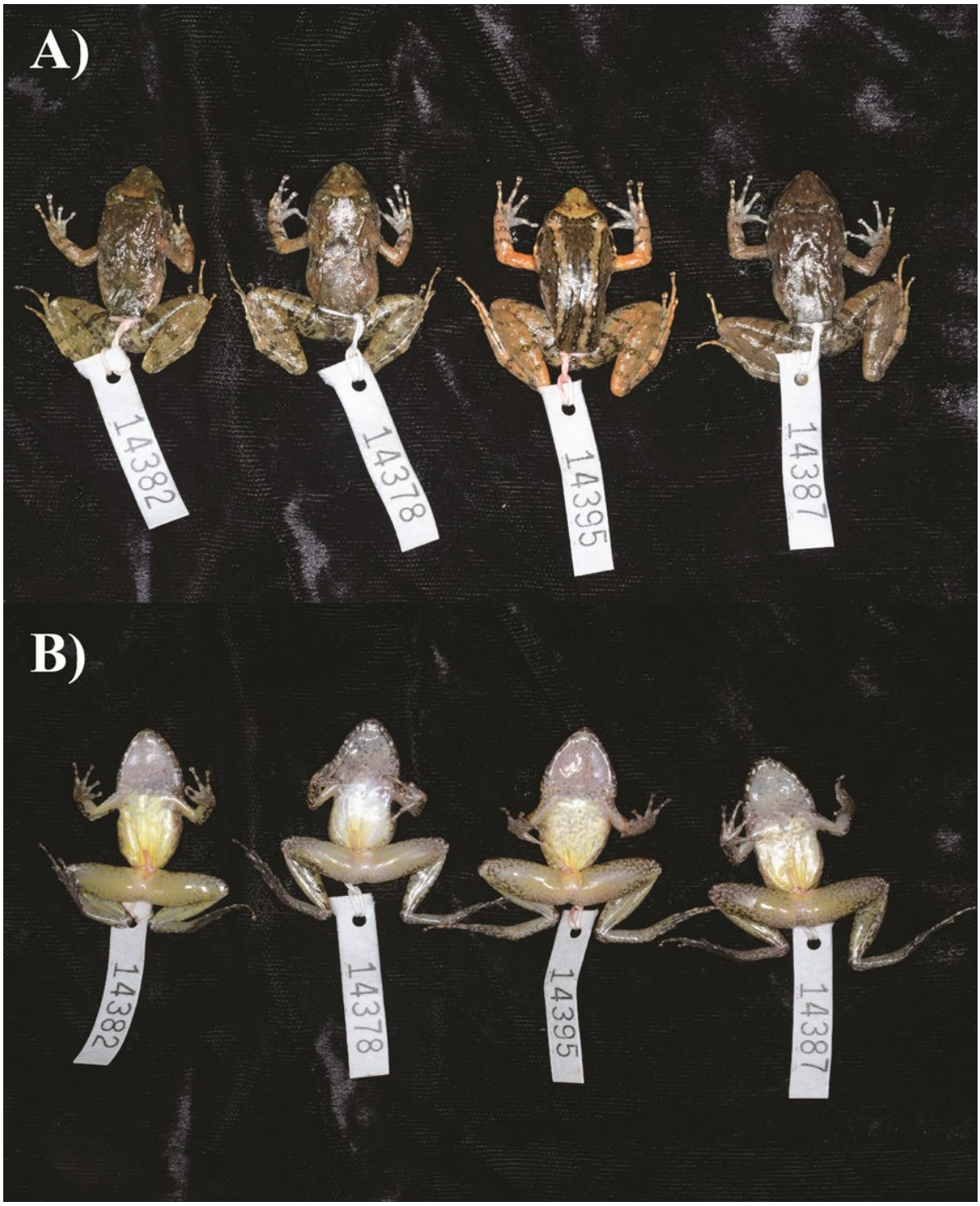

FIGURE 3—Images of a series of prepared *Limnonectes phyllofolia* showing dorsal and ventral color variation. The individuals depicted were collected on Gunung Bontosiri within Bantimurung National Park on 25 June 2014, at 592 m elevation.

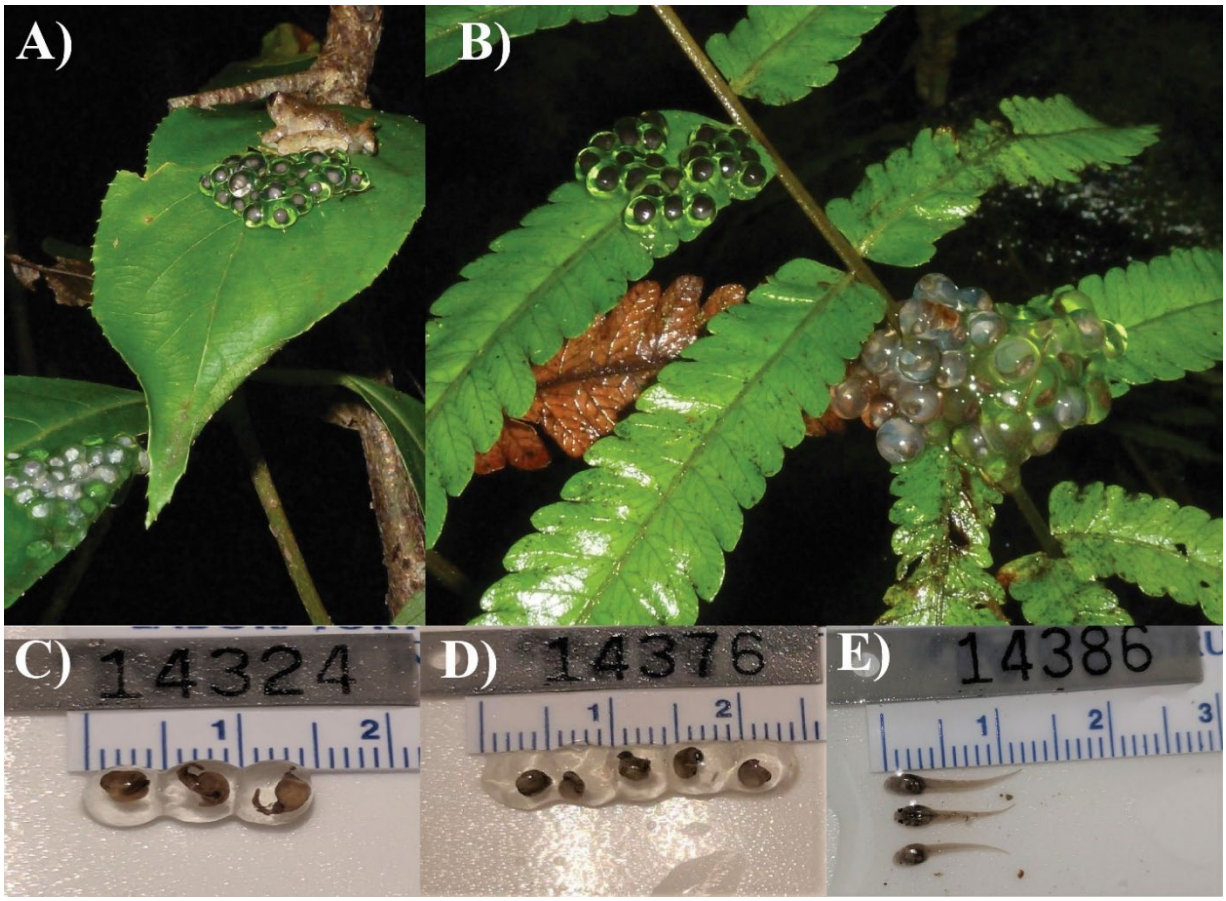

FIGURE 4—Eggs and newly hatched larvae of *L. phyllofolia*. (A) A male *L. phyllofolia*, JAM14375, guards two egg clutches on a sapling 2 m above a 1 m wide stream in Bantimurung National Park. (B) Example of dual egg clutches (guarded by JAM14324) deposited on fern frond 0.6 m above a puddle in Bantimurung National Park. (C) Example of eggs from clutch guarded by JAM14323 – clutch was collected from leaves 0.75 m above a puddle on 24 June 2014, 19:00 h from Bantimurung National Park. (D) Example of eggs from clutch guarded by JAM14375 – clutch was collected from leaves of a sapling tree, 2 m above a 1 m wide stream on 25 June 2014 at 21:38 h from Bantimurung National Park. (E) Example of newly hatched tadpoles. The associated clutch was guarded by JAM14385, and collected on a mossy boulder 1.5 m above a 1 m wide stream on 25 June 2014 at 21:38 h from Bantimurung National Park.

FIGURE 5—Ventral view of preserved male *L. phyllofolia* sp. nov. holotype showing palmer and plantar aspects of the hands and feet.

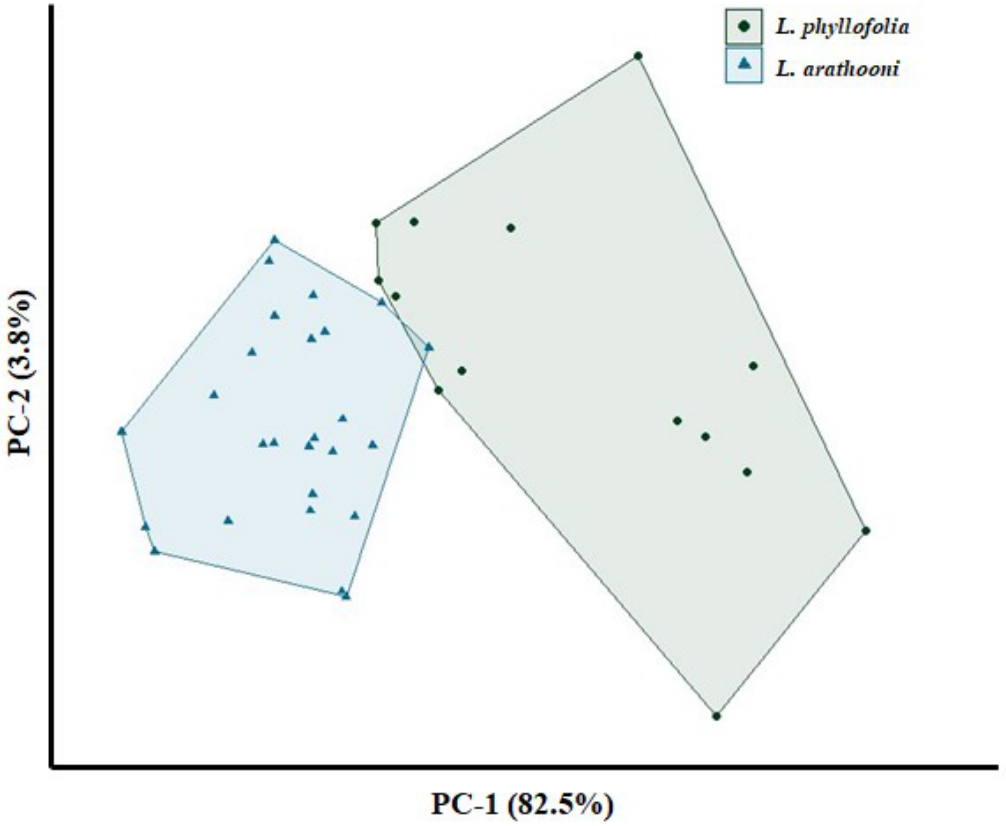

FIGURE 6—Results of principal components analysis on morphological characters. Biplot shows clustering of PC scores from morphological measurements by species. In PC-1, over 55% of the variance contribution within the model is attributed to the opposition (~5.5% each) of ten morphological characters: THL, LAL, HW, SVL, TL, FL, HAL, FLL, BW, and UAL. In PC-2, 88% of the variance contribution within the model is attributed to the opposition of five morphological characters: ED, TD, SN, ETD, SL.

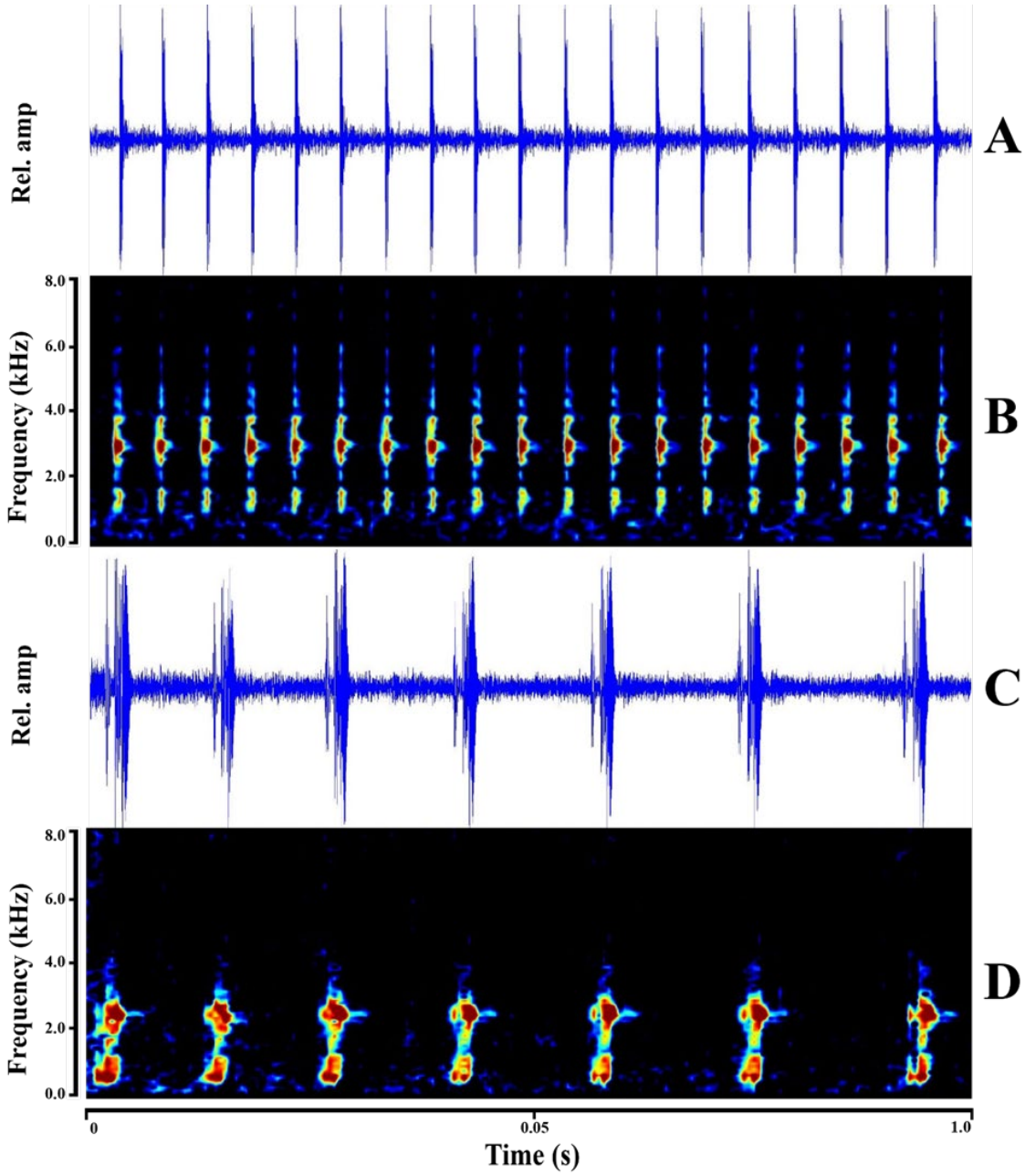

FIGURE 7— (A) Waveform oscillogram (relative amplitude vs. time in seconds) and corresponding (B) spectrogram (kilohertz vs. time in seconds) of a 19 note advertisement call of *L. phyllofolia* sp. nov. (JAM 14390) at Gunung Balease. The one second call was recorded from an approximate distance of one meter by J. A. McGuire on 27 June 2014, 23:30 h. (C) Waveform oscillogram and corresponding spectrogram (D) of a representative 7 note advertisement call of *L. arathooni* (JAM 14428), calling from a mossy boulder, 1 m above ground level, above a 1 m wide stream in Bantimurung National Park. The one second call was recorded from an approximate distance of 1 meter by J. A. McGuire on 25 June 2014, 22:02 h.

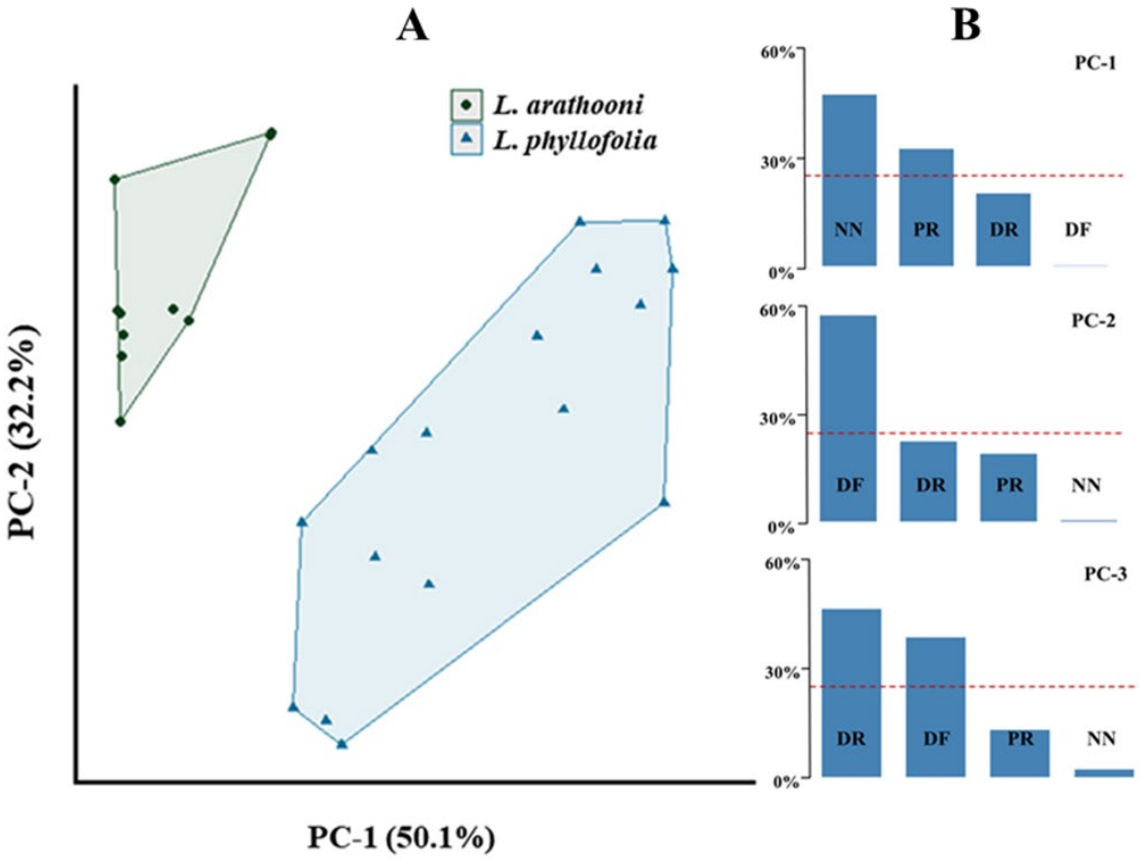

FIGURE 8—Results of principal components analysis on frog call characters. Biplot (A) shows clustering of advertisement call measurements by species. Bar charts (B) show the variance contributions of factors within the model, including: note number (NN), pulse rate (PR), call duration (DR), and dominant frequency (DF). Red dashed lines (B) demarcate values that contributed at least 25% of the overall variance to a PC.

Limnonectes phyllofolia morphological characters

	Males (27)		Females (2)		Juveniles (6)		Holotype
Head length (HL)	6.24—10.60	**(9.24)**	9.27—10.04	**(9.66)**	5.99—7.89	**(6.93)**	9.4
Head width (HW)	8.70—12.54	**(10.34)**	10.49—10.8	**(10.65)**	6.95—8.41	**(7.56)**	11.38
Snout-Vent Length (SVL)	21.53—30.13	**(27.03)**	26.70—29.29	**(28.00)**	18.13—22.23	**(19.67)**	28.12
Tibia Length (TL)	12.55—16.38	**(14.82)**	13.99—16.80	**(15.40)**	9.56—12.07	**(10.96)**	16.18
Interorbital Distance (IO)	1.91—3.12	**(2.67)**	2.56—2.86	**(2.71)**	1.94—2.47	**(2.18)**	2.98
Eye Diameter (ED)	3.04—4.25	**(3.50)**	3.01—4.00	**(3.51)**	2.71—3.08	**(2.82)**	4.14
Internarial Distance (IN)	2.05—2.89	**(2.50)**	2.38—2.55	**(2.47)**	1.70—2.29	**(1.92)**	2.58
Eye-Nostril Distance (EN)	1.94—2.96	**(2.44)**	2.58—2.71	**(2.65)**	1.66—2.25	**(1.98)**	2.58
Foot Length (FL)	12.07—16.41	**(14.25)**	12.08—15.85	**(13.97)**	4.70—11.96	**(9.42)**	15.48
Tympanum Diameter (TD)	1.34—2.82	**(2.12)**	2.25—2.30	**(2.28)**	1.28—2.13	**(1.62)**	2.56
Thigh Length (THL)	12.06—16.37	**(14.24)**	13.71—15.47	**(14.59)**	9.31—11.39	**(10.48)**	16.37
Snout Length (SL)	2.12—4.15	**(3.37)**	3.57—3.72	**(3.65)**	2.30—3.08	**(2.76)**	3.9
Hand Length (HAL)	6.13—8.81	**(7.59)**	7.79—8.35	**(8.07)**	4.41—5.80	**(5.21)**	8.15
Forearm Length (FLL)	4.38—6.28	**(5.34)**	4.98—6.43	**(5.71)**	3.00—4.60	**(3.97)**	5.25
Eye-Tympanum Distance (ETD)	0.62—1.54	**(1.03)**	1.02—1.09	**(1.06)**	0.52—0.72	**(0.64)**	1.21
Snout-Nostril Length (NS)	0.72—1.32	**(1.01)**	0.94—1.01	**(0.98)**	0.74—1.10	**(0.92)**	1.00
Upper Arm Length (UAL)	4.69—6.80	**(5.59)**	5.28—6.20	**(5.74)**	3.67—4.69	**(4.18)**	5.26
Lower Arm Length (LAL)	10.54—14.41	**(12.85)**	12.53—13.67	**(13.1)**	8.29—10.14	**(9.35)**	13.99
Body Width (BW)	7.14—11.11	**(9.13)**	8.24—8.99	**(8.62)**	5.79—7.59	**(6.68)**	9.78
Odontoid Process Length (OPL)	0.70—1.32	**(0.97)**	0.77—1.01	**(0.89)**	0.45—0.72	**(0.62)**	1.14
HL/SVL		**(0.34)**		**(0.34)**		**(0.35)**	0.33
HW/SVL		**(0.38)**		**(0.38)**		**(0.38)**	0.40
SL/SVL		**(0.12)**		**(0.13)**		**(0.14)**	0.14
EN/SVL		**(0.09)**		**(0.09)**		**(0.10)**	0.09
IN/SLV		**(0.09)**		**(0.09)**		**(0.10)**	0.09
ETD/SVL		**(0.04)**		**(0.04)**		**(0.03)**	0.04

OPL/SVL	**(0.04)**	**(0.03)**	**(0.03)**	0.04
TD/SVL	**(0.08)**	**(0.08)**	**(0.08)**	0.09
IO/SVL	**(0.10)**	**(0.10)**	**(0.11)**	0.11
ED/SVL	**(0.13)**	**(0.13)**	**(0.14)**	0.15
TL/SVL	**(0.55)**	**(0.55)**	**(0.55)**	0.58
THL/SVL	**(0.53)**	**(0.52)**	**(0.53)**	0.58
HAL/SVL	**(0.28)**	**(0.29)**	**(0.26)**	0.29
FL/SVL	**(0.53)**	**(0.50)**	**(0.48)**	0.55
HW/HL	**(1.12)**	**(1.10)**	**(1.10)**	1.21
SL/HW	**(0.33)**	**(0.34)**	**(0.36)**	0.34
IO/IN	**(1.07)**	**(1.10)**	**(1.15)**	0.86

TABLE 1—Range of Morphological character measurements (in mm) of adult *L. phyllofolia* sp. nov. paratypes (average given in parentheses), morphological character measurements of the holotype (in mm), and average SVL corrected measurements (in mm).

Limnonectes arathooni Measurements		
Morphological Character	**Adults (14)**	
Head length (HL)	9.91—14.51	(12.18)
Head width (HW)	12.86—17.54	(14.55)
Snout-Vent Length (SVL)	29.47—44.30	(35.23)
Tibia Length (TL)	14.90—24.35	(19.89)
Interorbital Distance (IO)	2.44—4.58	(3.46)
Eye Diameter (ED)	2.75—5.22	(3.85)
Internarial Distance (IN)	2.85—4.52	(3.55)
Eye-Nostril Distance (EN)	2.25—3.55	(2.88)
Foot Length (FL)	14.66—23.06	(19.15)
Tympanum Diameter (TD)	2.13—2.87	(2.54)
Thigh Length (THL)	14.81—24.44	(19.59)
Snout Length (SL)	2.12—4.15	(4.75)
Hand Length (HAL)	7.84—11.79	(9.99)
Forearm Length (FLL)	5.50—9.15	(6.96)
Eye-Tympanum Distance (ETD)	1.21—2.10	(1.58)
Snout-Nostril Length (NS)	0.97—1.90	(1.43)
Upper Arm Length (UAL)	5.90—8.72	(7.09)
Lower Arm Length (LAL)	13.70—19.00	(16.4)
Body Width (BW)	8.77—18.88	(12.73)
Odontoid Process Length (OPL)	0.98—2.08	(1.45)

TABLE 2—Range of body size and limb length measurements (in mm) of n=14 adult *Limnonectes arathooni* specimens used in our comparative morphological analyses (average given in parentheses).

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CHAPTER 2

Cryptic Diversity in South Sulawesi Fanged Frogs (Anura: Dicroglossidae): Molecular and Morphological Investigations Reveal Two Novel High-Elevation Species

ABSTRACT

The Indonesian island of Sulawesi represents a hotspot of global biodiversity and endemism, though unresolved degrees of morphological, ecological, and molecular disambiguation across amphibian lineages have led to dubious taxonomic assignments. Similar-looking animals within a given genus can be erroneously assumed to represent the same species; yet, they actually represent instances of cryptic speciation. Elucidating and diagnosing cryptic species are thus critical needs with regard to the aims of assessing biodiversity for the purposes of both species- and systems-level conservation efforts. Herein, we report two fascinating cases of cryptic speciation in South Sulawesi *Limnonectes* and characterize two novel high-elevation species based on morphological, molecular, and acoustic evidence. In both cases, the new fanged frogs can be diagnosed from their low-elevation analogs, *Limnonectes arathooni*- and *Limnonectes microtympanum* on the basis of body size, genetic distance, demography, and advertisement call.

INTRODUCTION

A central tenet of evolutionary- and conservation biology is the need to understand drivers of faunal diversity. In the face of global change, this need is underscored within the geospatial band that contains the highest diversity while also being the most imperiled: the rainforests of the equatorial tropics. Indeed, one way to qualify the biological significance of these globally threatened regions is to prioritize hotspots of endemism: those areas that contain a high richness of organisms that exist nowhere else on Earth (Myers, et al. 2000). Though the biological significance of endemism hotspots has been documented, often, these regions house enigmatic assemblages of organisms that have yet to be described in the scientific literature (Hansen et al. 2013; Lohman et al. 2011; Myers, et al. 2000).

The Indonesian island of Sulawesi represents one such hotspot of global biodiversity and endemism (Iskandar et al. 1998; Iskandar and Tjan 1996; Lohman et al. 2011; Mittermeier et al. 1999; Myers et al. 2000; Von Rintelen et al. 2012; Whitten and Henderson 2012). The island is topographically rugged – boasting many mountains over 5000 meters (m) in elevation. As Sulawesi is also equatorial, the island houses an array of lowland rainforest that grades to upland montane rainforest, mossy cloud forests, and alpine scrub habitats. Though the biological significance of Sulawesi taxa was noted by Alfred Russel Wallace as far back as the 1800's, in recent years, investigators have not only identified a preponderance of new species on Sulawesi, but several assemblages representing remarkable radiations of novel endemic: rats (Muridae), shrews (Eulipotyphla), frogs (Dicroglossidae), lizards (Agamidae), shrimp (Atyidae), snails (Pachychilidae), begonias (Begonaceae), and carnivorous pitcher plants (Nepenthaceae) (Ardi et al. 2018; Esselstyn et al. 2021; Handika et al. 2021; McGuire et al. 2022; Murphy et al. 2020;

Rowe et al. 2016; Setiadi et al. 2011; Thomas et al. 2011; Von Rintelen et al. 2012; Wallace 2013).

An ongoing challenge in attempting to assess the extent of radiative diversity in a hotspot of endemism like Sulawesi, is that mechanisms underpinning evolution and lineage divergence are often varied and clade-specific. Speciation scenarios may play out under a variety of biotic or abiotic conditions, producing: (1) assemblages that are relegated to particular areas of endemism or ecotypes (e.g., crabs, monkeys, tarsiers, flying lizards, and toads), (2) convergence in eco-morphology (e.g., freshwater snails and silverside fish), and (3) explosive radiations that could be the result of any number of mechanistic permutations involving introgression, classical allopatry, isolation by distance or environment, incomplete lineage sorting, and / or competitive replacement (e.g., Lake Malili shrimp, fanged frogs, and white-toothed shrews) (Esselstyn et al. 2021; Evans et al. 2003; Setiadi et al. 2011; Von Rintelen and Cai 2009; Von Rintelen 2011; Von Rintelen et al. 2012). Scientists often misclassify similar-looking animals; thus, large species complexes are grouped together taxonomically. This tendency is exacerbated by unresolved degrees of morphological, ecological, and molecular disambiguation across species. Indeed, there is a critical need for an increasing the number of biological studies that seek to elucidate cryptic speciation events, especially those aiming to quantify both the overall extent and mechanistic drivers of biodiversity (Sheridan and Stuart 2018).

Amphibians represent archetypal organisms with which to uncover cryptic speciation in this region, and among them, the Dicroglossid fanged frogs of genus *Limnonectes* have been the subject of increased interest in recent years (Bain et al. 2007, Brown and Iskandar 2000; Evans et al. 2003A; Inger and Stuart 2010; Iskandar et al. 2014; Kusrini et al. 2015; Mcleod 2010; Mcleod et al. 2011; Reilly et al. 2019; Rowley et al. 2010; Setiadi et al. 2011; Ziegler and Wu 2018). Both cryptic speciation and the existence of large species complexes were well-documented in the 1990's and early 2000's by the pioneering works of Djoko Iskandar and Sharon Emerson, especially regarding *L. blythii*, *L. kuhlii*, and *L. finchii* (Emerson and Inger 1992; Emerson and Berrigan 1993; Emerson 1994; Emerson 1996; Emerson 2001; Iskandar and Tjan 1996; Iskandar 1998; Iskandar et al 1996; Mcleod et al. 2011). Since then, several new species have been described, and further, yet-unresolved complexes (especially on Sulawesi) have been identified (Aowphol et al. 2015; Frederick et al. 2022; Iskandar et al. 2014; Kohler et al. 2021; Setiadi et al. 2011; Ye et al. 2007; Yodthong et al. 2021).

In 2016, as part of a comprehensive study to resolve the systematics of Sulawesi *Limnonectes*, we conducted biological inventory surveys of fanged frogs on Sulawesi's Southwest Peninsula. Herein, we report two fascinating cases of cryptic speciation and characterize two novel high-elevation species based on morphological, molecular, and acoustic evidence. In both cases, the new frogs can be diagnosed from their low-elevation analogs, *L. arathooni*- and *L. microtympanum* on the basis of body size, genetic distance, demography, and advertisement call. One of the novel species is also now only the third exemplar in the vast Sulawesi assemblage known to be a non-aquatically breeding outlier: exhibiting both terrestrial female egg deposition and male parental care behaviors (Brown and Iskandar 2000, Frederick et al. 2022).

MATERIALS AND METHODS

Field Sampling .— In collaboration with Indonesian Institute of Sciences (LIPI) - Museum Zoologicum Borgoriense (MZB) and Institut Teknologi Bandung (ITB), we conducted several expeditions between 2007 and 2016 to collect amphibians on Sulawesi's Southwest Peninsula. All research permits and associated permissions were facilitated by the Indonesian Ministry of Research, Technology, and Higher Education (RISTEK). Geographically, we focused our survey efforts on the following regions: Desa Bontomaranu, Bontosiri, the Lompobatang-Bawakaraeng mountain complex, and Desa Cikoro near the base of Gunung Lompobatang. In all cases we hand-captured frogs in the field and removed liver tissue samples (subsequently stored in RNA Later) during specimen preparation. Specimens were formalin fixed, thereafter stored in 70% ethanol, and deposited at either MZB or the UC Berkeley Museum of Vertebrae Zoology (MVZ). All animal handling and field project protocols were pre-approved by the UC Berkeley Institutional Animal Care and Use Committee (Protocol : R279).

Morphological Measurements.—We measured the following 20 morphological characters to the nearest 0.01 mm using digital calipers: head length (HL); head width (HW); snout-vent length (SVL); tibia length (TL); interorbital distance (IO); eye diameter (ED); internarial distance (ID); eye-nostril distance (EN); foot length (FL); tympanum diameter (TD); thigh length (THL); snout length (SL); hand length (HAL); forearm length (FAL); eye-tympanum distance (ETD), snout-nostril length (SNL); upper arm length (UAL); lower arm length (LAL); and body width (BW) following Watters et al. (2016), and one additional character (odontoid process length (OPL)). We calculated digital webbing formulae according to Guayasamin et al. (2006) and Stuart et al. (2020), whereby: fingers and toes were represented by Roman numerals and Arabic numerals correspond to the attachment position of the webbing on each of the respective phalanges.

Morphological Comparisons.—Presented with instances of cryptic speciation among sister-species, we considered it pragmatic to take a "dual-pronged" approach in our treatment of the morphometric data. Moreover, relative body sizes and limb lengths may exhibit both individual- and site-based variation. For these reasons, first employed empirical orthogonal functions in tandem with multivariate analysis of variance (MANOVA) tests on sister species pairs following (Frederick et al. 2022). This allowed for a global assessment of difference across all characters while mitigating the potential for violating variance-based model assumptions as they pertain to statistical non-independence, pseudoreplication, homoschedasticity, and spatial auto-correlation. In each case, we then performed variance component analysis by extracting the scores from the first three orthogonal dimensions and calculating the percent variance attributed to each PC by various morphological characters.

Secondly, we aimed to explicitly test for statistically significant differences between individual morphological characters between species based on the orthogonal variance components results to corroborate or contradict any fine-scale differences detected by the first approach. Upon identification of these high variance component characters, we used our original (non-transformed) morphological measurements for to perform iterative Mann-Whitney U tests to identify any statistically significant differences between individual characters for each sister species pair.

Acoustic Sampling.—During our collecting expeditions, we opportunistically collected breeding call data from various *Limnonectes*. Upon discovery of individuals engaged in advertisement behavior, we recorded calls using a solid-state recorder (Marantz Professional, USA: PMD661MKII) fitted with a shotgun condenser microphone (Sennheiser: MKH 60-P48). We isolated individual calls using Raven Pro Sound Analysis Software (v. 1.6.3 – Bioacoustics Research Program, 2014) that calculated both spectrogram- and waveform views using a Fast Fourier Transformation of 512. For each call, we recorded note number, call duration (in seconds), pulse rate (notes per second), and mean dominant frequency in kilohertz (kHz).

Genetic Sampling.—We first obtained genomic DNA from preserved liver tissue samples of *L. microtympanum* and *L. arathooni* specimens via salt extraction. We then amplified: a 390–400 base pair (bp) fragment of the 12S ribosomal RNA (rRNA) mitochondrial marker with primers H1478 (5'-TGACTGCAGAGGGTGACGG-GCGGTGTGT-3') and L1091 (5'-AAAAAGCTTCAAACTGGGATTAGATACCCC-ACTAT-3'); a 515–530 bp fragment of the 16S rRNA marker with primers 16S-H3062 (5'-CCGGTTTGAACTCAGATCA-3') and 16SB-FROG (5'-CGCCTGTTACCAAAAACAT-3'); and a 580 bp fragment of the cytochrome c oxidase (CO1) marker using the cocktail of primers VF1 (5′-TTCTCAACCAACCACAAAGACATTGG-3′), VF1d (5′-TTCTCAACCAACCACAARGAYATYGG-3′), VF1i (5′-TTCTCAACCAACCAIAAIGA-IATIGG-3′), VR1 (5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′), VR1d 5′-TAGACTTCTGGGTG-GCCRAARAAYCA-3′, and VR1i (5′-TAGACTTCTGGGTGICCIAAIAAICA-3′) described by Ivanova et al. (2006). Polymerase chain reaction (PCR) conditions were the same for all three genes: denaturation at 94°C – 2 min, 35 cycles (denaturation at 94°C – 45 s, annealing at 53°C – 30 s, extension at 72°C – 1 min), and final extension at 72°C for 1 min. We purified all amplicons with ExoSAP-it (Applied Biosystems) and performed cycle sequence reactions using the respective amplifying primer sets and BigDye v 3.1. We further purified our cycle sequence products using Sephadex G-50 and sequenced the samples on an ABI 3730 automated DNA sequencer (Applied Biosystems). Lastly, we edited and manually aligned all sequences in Geneious 9.1.8 (Biomatters).

Upon identification of putative species using our Sanger data, we further sampled our genomic data using next-generation sequencing (NGS) for 22 *L. arathooni*, 32 *L. microtympanum*, 9 *L. phyllofolia*, and single representatives of *L. heinrichi*, *L. larvaepartus*, and *L. modestus* using two approaches. First, we obtained low-coverage complete genome data by generating library preparations (performed by Daicel-Arbor Biosciences) and then sequencing them directly on the Illumina HiSeq2500 platform with 150 bp paired-end reads. From these data, we ultimately collected complete mitochondrial genome sequences as described below. We also performed a targeted capture experiment using the same library preps together with the FrogCap modular sequence capture system, screening a subset of markers from the "Ranoidea" probe-set known to perform well with *Limnonectes* (Hutter et al. 2022). This probe-set included 7247 target loci (mean length of 372 bp), which included 961 transcriptome-derived markers developed for Lesser Sundas *Limnonectes* (see Reilly et al. 2019). The baits were 120 bp in length with 2X tiling density. Both the low-coverage genome data and the sequence-capture data were cleaned and filtered using the FrogCap bioinformatics pipeline (Hutter et al. 2022; https://github.com/chutter/FrogCap-Sequence-Capture). Using this pipeline, raw Illumina reads were de-multiplexed using the Illumina software Bcl2fastq. Raw reads were cleaned of adaptor contamination, low complexity sequences, and other sequence artifacts using FASTP (Chen et al.

2018). Adapter-cleaned reads were decontaminated by mapping to contaminant genomes with BBMAP (part of BBTools; https://jgi.doe.gov/data-and-tools/bbtools/). Cleaned paired-end reads were merged and missing gaps were filled using the BBMerge function (Bushnell et al. 2017) in BBTools. Exact duplicates were then removed using the "dedupe" function of BBTools. Cleaned reads were assembled using SPADES v.3.12 (Bankevich et al. 2012), which ran BAYESHAMMER (Nikolenko et al. 2013) error correction. SPADES used several k-mer values (21, 33, 55, 77, 99, 127). DIPSPADES (Sofanova et al. 2015) was used to generate consensus sequences from polymorphic contigs. For the exon-capture data, consensus haplotype contigs were then matched to our probe set reference sequence file using BLAST (*dc-megablast*), with contigs discarded if they failed to match ≥30% of the reference marker or 50 bp of the reference marker. Markers retained after filtering were aligned one-by-one using MAFFT (Katoh and Stanley 2013). Each alignment was screened for samples ≥ 40% divergent from the consensus, and such samples were excluded. Alignments were separated into two initial data sets corresponding to "Exons-Only" (exon contigs with introns trimmed off), and "All-Markers" (which included the entire matching contigs, including UCEs). The Exons-Only and All-Markers alignment sets were then re-processed to generate a data set ("gene-all-markers trimmed") composed of individual genes for which all sequenced exons and introns were concatenated.

SNPs were called from sequence capture loci using R scripts that are part of the FrogCap bioinformatics pipeline and available at https://github.com/chutter/FrogCap-Sequence-Capture. The pipeline uses GATK v4.1 (McKenna et al. 2010) following developer best practices to discover and call SNP variants. The procedure involves creating a reference sequence for each gene using a consensus sequence from each alignment from the target group (in our case, *L. arathooni*, *L. microtympanum*, or *L. phyllofolia*), and then using BWA (Li 2013) to map the cleaned reads from each sample back to that reference while adding the read group information (e.g., Flowcell, Lane, Library) obtained from the fastq header files. SAMTOOLS (Liu et al. 2009) was used to convert the mapped reads SAM file to a cleaned BAM file, and to merge BAM files with unmapped reads. PICARD was used to mark exact duplicate reads that may have reflected optical or PCR artifacts and to reformat each data set for variant calling. To identify variant and invariant sites, the GATK program *HaplotypeCaller* was used to call haplotypes in GVCP format for each individual sample. For each sample, the GATK program *GenomicsDBImport* was used to aggregate samples from the separate datasets into their own combined database. With these databases, the GATK function *GenotypeGVCF* was used to combine sample data sets and output separate ".vcf" files for each marker containing variant data for final filtration. From this preliminary variant set, one high quality SNP per locus (quality > 20) was retained for downstream analyses, with a custom R script used to produce STRUCTURE (Pritchard et al. 2000) input files.

We also used the FrogCap bioinformatics pipeline to pull complete mitochondrial genome sequences from the low-coverage genome data set. The pipeline uses the same approach as described above for the modified Ranoidea FrogCap probe-set, but maps the cleaned Illumina reads to the mitochondrial genome reference file for *Nanorana parkeri*. The final alignment was 18,315 bp in length.

Phylogenetic Estimation and Demographics.—We performed a maximum likelihood phylogenetic analysis of the mitogenome data set using IQTree. The data set was concatenated under a single partition and a GTR+I+G nucleotide substitution model of evolution was selected for the analysis with 1000 ultrafast bootstrap replicates.

Separate STRUCTURE analyses were undertaken for *L. arathooni*, *L. microtympanum*, and *L. phyllofolia*, each using one SNP per gene. For *L. arathooni*, this corresponded to 5845 SNPs, for *L. microtympanum,* we analyzed 5849 SNPs, and for *L.* leaf-nester, we analyzed 5802 SNPs. For each data set, the program was run for 500,000 generations as burn-in, followed by 1,000,000 stationary generations. We ran K=2-3 for *L. arathooni*, K=2-4 for *L. microtympanum*, and K=2-4 for *L. phyllofolia*. The results were then imported into structure harvester (Earl 2012) to determine the most likely number of populations as determined by both the Delta K method and highest mean estimate of the Ln probability of the data.

Nomenclature acts.—This article adheres to the International Code of Zoological Nomenclature (ICZN). All nomenclature acts within are registered within the ICZN system on Zoobank.org. The Zoobank Life Science Identifier (LSID) for this published work and its associated nomenclature acts can accessed online at https://www.Zoobank.org, reference codes: urn:lsid:zoobank.org:pub:742C07C8-FD5C-4BEE-954C-89706E2C0A9F, and urn:lsid:zoobank.org:act:E746F904-CA5E-47F1-9CD6-FC249D4004AC.

The new high-elevation sister species to *L. arathooni* may have been inadvertently collected in from Gunung Lompobatang 1924 by Malcolm A. Smith and referenced as paratypes of *Rana arathooni* in Smith (1927). Though precise coordinates of the original collection site are unavailable, Smith's *R. arathooni* paratype specimens deposited at the Harvard Museum of Comparative Zoology as A-13386–8 could potentially be misidentified exemplars of the new species. This remains unclear without genetic confirmation, and as such, specimens from subsequent collecting expeditions on Sulawesi that were deposited at the British Natural History Museum under the *Rana arathooni* moniker could potentially include exemplars of the new species. Lastly, specimens of both *L. arathooni* and the new species were undoubtedly collected by R. Brown and D. Iskandar and referred to as *L. arathooni* in Brown and Iskandar (2000). Importantly, catalog records from the Museum of Comparative Zoology indicate the post hoc assignment of coordinates to the *L. arathooni* type-locality as the peak of Gunung Lompobatang, rather than the actual site of original collection by Smith (1927). Thus, we clarify the designation of *L. arathooni* herein as the low elevation species (Smith listed the *L. arathooni* type locality as Desa Cikoro: a village at the base of the mountain massif) and subsequently diagnose the high-elevation form with this manuscript.

The new high-elevation sister species to *L. microtympanum* was likely referenced as *Rana microtympanum* in Boulenger (1920), and by implication as: *Dicroglossus microtympanum* by Deckert (1938), *Rana* (*Euphlyctis*) *microtympanum* by Dubois (1981), *Euphlyctis microtympanum* by Poynton and Broadley (1985), and Dubois (1987). The original description of *Rana microtympanum* (now *L. microtympanum*) by Van Kampen (1907) leaves nebulous uncertainty as to the true identification of the originally collected specimens. The collection locality of the *Rana microtympanum* type-specimens was heretofore described as occurring on Gunung Lompobatang above 1000 meters = encompassing the range of both sister species (Van Kampen 1907). Thus, we clarify the designation of *L. microtympanum* herein as the low elevation species and subsequently diagnose the high elevation form within this manuscript.

RESULTS

Limnonectes ***diatas*** **sp. nov**.

(Figures 2A, 4B)

Etymology.—As the new fanged frog is a high-elevation analog of its sister species *L. arathooni*, we chose the specific epithet, *"diatas*": derived from the Bahasa Indonesia term *di atas* – meaning "above" or "atop".

Holotype.—An adult male (JAM 15135), collected 30 Oct 2016 at 21:02 h, from Sulawesi Island, Indonesia: Sulawesi Selatan Province: Kabupatan Sinjai: Kecematan Sinjai Barat: Desa Gunung Parak: Gunung Bawakaraeng (S 05.28399, E 119.95911 ± 7 m) at 1738 m by J. H. Frederick and J. A. McGuire.

Paratypes.—JAM 14912, an adult male collected on 13 October 2016 at 1702 m elevation; JAM 14946, an adult male collected on 16 October 2016 at 1677 m elevation; JAM 14987–8, one egg mass and one adult male collected on 17 October 2016 at approximately 1700 m elevation; JAM 15044–5, two adult males collected on 20 October 2016 at 1715 m elevation; JAM 150101–4, three adult males and one adult female collected on 24 October 2016 at 1709 m elevation; JAM 15110, an adult male collected on 25 October 2016 at 1708 m elevation; and JAM 15172–80, five egg clutches and four adult males collected on 2 November 2016 between 1682- and 1696 m elevation. All paratypes were collected by J. H. Frederick, J. A. McGuire, W. Triloksono, and H. Rockney.

Distribution.—*Limnonectes diatas* sp. nov is endemic to the island of Sulawesi and, to our knowledge, restricted to the Southwest Peninsula south of the Tempe Depression (FIGURE 1A). Though we cannot fully predict the complete species range South of Tempe, we have high confidence that it occurs where appropriate upland stream habitats exist above 1500 m in elevation throughout Banitmurung National Park (FIGURE 1C). Outside of this predicted range in Bantimurung however, *L. diatas* sp. nov is only definitively known from upland habitats within the Lompobatang-Bawakaraeng mountain complex (FIGURE 1B). In 1998, five specimens of *L. diatas sp. nov* were collected as part of another study on the south side of Gunung Lompobatang by R. Brown and D. J. Iskandar - at an elevation of ~1600 m. The type locality for this species was later defined in 2016 through extensive surveys on the north face of Gunung Bawakaraeng by J. Frederick and J. A. McGuire - at an elevational range between 1682–1719 m.

Diagnosis.—*Limnonectes diatas* sp. nov can generally be found living in sympatry/syntopy with the high-elevation sister species to *L. microtympanum* (diagnosed below). Though we have identified a crudely defined contact zone on Gunung Bawakaraeng in the form of a 120 m elevational band between ~1500–1670 m demarcating species turnover between *L. arathooni* and *L. diatas* sp. nov, further investigations are needed to verify the consistency and elevational extent of species turnover across other mountain habitats on South Sulawesi. As the newly described *L. phyllofolia* appears to be obligate to forest below 1200 m (Frederick et al. 2022), we think it highly unlikely that these two species could be found to co-occur on the Southwest Peninsula. In adjacent lowland forest, however, *L. microtympanum* and *L. arathooni* are likely common.

Outside of the aforementioned fanged frog species, *L. diatas* sp. nov can be easily distinguished from all other Sulawesi congeners on the basis of geographic range, being solely restricted to the Southwest Peninsula south of the Tempe Depression (FIGURE 1C). Though the full suite of reproductive modes of the Sulawesi fanged frog assemblage have not yet been elucidated, *L. diatas* sp. nov is only the third species (after *L. arathooni*) known to terrestrially deposit clutches of eggs on mossy stream banks less than one meter from water, whereafter males subsequently exhibit nest guarding behavior until tadpoles emerge. Regarding its only verified sympatric congener, *L. diatas* sp. nov can be easily distinguished from the high-elevation sister to *L. microtympanum* based on advertisement call (see acoustic analyses below) and body size, being substantially smaller than the upland *L. microtympanum* analog (*L. diatas* sp. nov average SVL: 30.68 mm, range: 26.20– 37.82; *L. kejutani* sp. nov average SVL: 67.68 mm, range: 60.19–77.66). Unlike its larger sympatric congener that utters a guttural "quack!", *L. diatas* sp. nov utters a high-pitched, "chirp"-like advertisement call that cannot be confused with the former.

Description of holotype.—An adult male (FIGURE 2A, 4B), SVL 32.72 mm; body squat; head large and wide especially relative to both overall body size- and the widest span of the trunk: HL 1.22 mm, HW 12.86 mm, 32.18% and 39.30% of SVL, respectively; moreover, head is wider than long, 122.12% of HL, with HW in life roughly equivalent to BW (HW/BW = 113.60%, in paratypes $\bar{x}$ =125.86%); interorbital distance roughly equal to internarial distance (100.60%), with both characters being: (1) definitively longer than the distance from the anterior margin of the eye to the nostril (127.02%), and (2) longer than the diameter of the tympanum (154.46%); distance from the posterior margin of the eye to the anterior margin of the tympanum (ETD) is notably shorter than both TD and END (65.73% and 54.05%, respectively); eye large, round, and unimpeded, 3.21 mm in diameter, 9.81% of SVL and 30.48% of HL, with quadrilateral pupil and metallic gold iris; interorbital distance 3.29 mm, with the interorbital span laying flat across the fronto-parietal, exhibiting no notable convex boss; tympanum round, 2.13 mm in diameter, only slightly hooded infra-anteriorly by a supra-tympanal skin fold that initiates at the posterior margin of the eye, extending over and around the tympanum, terminating just above the arm; odontoids small (1.16 mm), lacking prominence, much like the absence of hypertrophied postorbital musculature found across many large-bodied fanged frog congeners.

Arms stocky, held wide and tall beneath the trunk, with FAL only slightly shorter but roughly equivalent to UAL (92.00%), lending a notably alert, distinguished appearance to the animal; distance from the wrist to the tip of finger IV (HNDL) 32.18% of SVL and 92.59% of HL, being reliably longer than both FAL and UAL (176.09% , 162.00%, respectively); fingers short and unwebbed, bowed inward such that the thumbs point slightly backward toward the clavicles, with relative finger lengths **III > I > II > IV (FIGURE 2A, B, D; 4B)**; hindlimbs long, with THL, TL, and FL roughly equal (THL 98.4% and 97.12% of TL, FL, respectively; in paratypes, $\bar{x}$ THL = 102.05% and 103.92% of TL and FL, respectively); toes short save for digit IV, with hind foot webbing moderately reduced and webbing formula: **I** 0^{+}–1^{-} **II** 0–1 **III** 1–3^{-} **IV** 3^{+}–2 **V** (FIGURE 4B); dorsal skin rugosity primarily limited to the flanks; dorsolateral dermal plicae prominent anteriorly — initiating post-orbitally, though may appear to dissolve midway down the trunk about the ilium.

Coloration.—Especially in life but also in preservative, prominent dorsal markings include a stark chocolate brown mask that initiates at the snout and fully spans the nostril, subsequently bisecting the eye, terminating beneath the tympanum just above the posterior margin of mouth (FIGURE 2A, D; 4B). Dark lip bars, banding across the thighs and tibia, and (more-subtle) arm

bands of the same color are also present. A transverse brown bar runs across the head in between the upper eye lids, though the shade of the bar may vary across individuals in life. Eyes are metallic gold with rhomboid pupils, though in some the gold may grade to a dark bronze or brown where the mask bisects the eye. Generally, live frogs exhibit either a barred or standard morph, with barred individuals tending to have a darker base coloration and gold or cream-colored bars initiating behind the eyes and running posteriorly toward the urostyle, tracing the margins of the dermal plicae (FIGURE 2D). Standard morph individuals may range in color from cinnamon, umber, or dark chocolate brown, and may also appear to have a slightly brick-red appearance (FIGURE 2A, D). Often, dorsolateral dermal plicae on both morphs appear red in life, though this quickly disappears in preservative (FIGURE 4B). Small, dark brown spots or blotches may be present anywhere on the dorsum, though are never regular or patterned where they exist (especially on the snout). Fingers and toes have a marbled appearance. In life, the ventral side of the animal is mottled across the trunk, though this usually grades out anteriorly toward the mouth. There is a distinct sunflower yellow wash spanning the undersides of the arms, legs, feet, and posterior-half of the trunk; however, its prominence reliably fades in preservative (FIGURE 2B).

Reproductive Observations.—We collected four terrestrial nests of *L. diatas* sp. nov eggs (identified in each case by the presence of an attending male). Each had ~30 very large, firm, spherical eggs approximately 8 mm in diameter containing a mostly translucent jelly. Golden clay-colored embryos could be clearly observed within each capsule. As is commonplace with many terrestrially nesting anuran species, close contact by guardian males almost certainly includes provisioning the egg clutches, in effect, preventing fungal growth and infestation, and keeping the eggs from rotting (e.g., see Simon 1983) (FIGURE 2C). Similar to other terrestrially nesting fanged frog species we've observed on Sulawesi (e.g., *L. phyllofolia* and *L. arathooni*), male *L. diatas* sp. nov appear to guard 2-3 nests in close proximity and will continue advertisement calling even when attending nests. Each nest was found adjacent to a 0.5—2 m wide, slow-flowing stream and deposited 0.7—1.7 m up a ~70 degree sloped, mossy bank (FIGURE 2C, 5C). As was also described by Brown and Iskandar (2000) in *L. arathooni*, we are confident that tadpoles emerge from their eggs and wriggle down their natal stream banks into the water below. While collecting *L. diatas* sp. nov nests for this project, we observed that prematurely-developed tadpoles emerged from the egg capsules immediately upon placing the eggs in vials of salt, alcohol, or formalin preservative. Presumably, this somewhat common response for terrestrially nesting frog larvae (see Brown and Alcala 1982; Brown and Iskandar 2000, Warkentin 1995; Warkentin 1999) is an anti-predator behavior to bolster larval survival.

Variation.—Outside of the aforementioned potential for variable dorsal coloration in life, *L. diatas sp. nov* seems to vary little morphologically across the specimens we've observed. That said, both male- and female-based sexual dimorphism occurs in *Limnonectes* throughout their range; thus, we cannot rule out the possibility of slight body-size differences between the sexes. More specimens will need to be observed and measured in the field to confirm or deny this possibility.

Habitat Description.— Both species diagnosed herein are found in sympatry along upland stream networks. See the comprehensive habitat description for both species following the subsequent diagnosis.

Limnonectes ***kejutan*** **sp. nov**

(Figs. 3C, 4A)

Etymology.—We originally purported the potential for a new cryptic species while observing peculiar call differences in *L. microtypmanum* at differing elevations on Gunung Bawakaraeng in 2016. Due to the wholly unexpected finding of high—low sister species analogs, we chose the specific epithet, "*kejutan"* – from the Bahasa Indonesia word meaning "surprising".

Holotype.—An adult male (JAM 14899), collected 13 Oct 2016 at 22:25 h, from Sulawesi Island, Indonesia: Sulawesi Selatan Province: Kabupatan Sinjai: Kecematan Sinjai Barat: Desa Gunung Parak: Gunung Bawakaraeng (S 05.28419, E 119.25802 ± 13 m) at 1738 m by J. H. Frederick and J. A. McGuire.

Paratypes.—JAM 14895–14898, 14900–14911, 14915, 14916 [larvae], eighteen adult males and one tadpole lot, collected on 13 October 2016 between 1703- and 1738 m elevation; JAM 14933–6, 14955, 14961, six adult males, collected on 14 October 2016 between 1579- and 1712 m elevation; JAM 14973, 14975–7, four adult males collected on 17 October 2016 at approximately 1703 m elevation; JAM 15048–9, one adult female and one adult male, collected on 20 October 2016 at approximately 1703 m elevation; JAM15074–80, three adult females and four adult males, collected on 22 October 2016 at approximately 1550 m elevation. All paratypes were collected by J. H. Frederick, J. A. McGuire, W. Triloksono, and H. Rockney.

Distribution.— *Limnonectes kejutan* sp. nov is endemic to the island of Sulawesi and restricted to the Southwest Peninsula south of the Tempe Depression. As with its sympatric congener described above, we cannot fully predict the complete species range, though it likely occurs where appropriate upland stream habitats exist above 1500 m in elevation throughout Banitmurung National Park. *L. kejutan* sp. nov is definitively known from two localities: the first being the Lompobatang-Bawakaraeng mountain complex, and the second being Gunung Bontosiri within Bantimurung National Park. In 1998, several specimens of *L. kejutan* sp. nov were collected as part of another study on the south side of Gunung Lompobatang by R. Brown and D. J. Iskandar — at an elevation of 1580 m, assuming them to be *L. microtympanum*. The type locality for this species was later defined in 2016 through extensive surveys on the north face of Gunung Bawakaraeng by J. Frederick and J. A. McGuire - at an elevational range between 1485—1738 m.

Diagnosis.—*Limnonectes kejutan* sp. nov can be found living in sympatry / syntopy with its much smaller high-elevation congener *L. diatas* sp. nov (diagnosed above). Though we have identified a crudely defined contact zone on Gunung Bawakaraeng in the form of a 110 m elevational band between ~1370—1480 m demarcating species turnover between *L. microtympanum* and *L. kejutan*, further investigations are needed to verify the consistency and elevational extent of species turnover or possible contact zones across other mountains on South Sulawesi. As a high elevation obligate, *L. kejutan* sp. nov almost certainly would never co-occur with the only other fanged frog from the Southwest Peninsula, *L. phyllofolia* (a lowland obligate). That aside, it can be easily distinguished from *L. phyllofolia* based on body size, as *L. phyllofolia* is the smallest of all Sulawesi fanged frogs (see SVL comparisons in *L. diatas* sp. nov diagnosis above). Outside of the comparisons between *L. microtympanum*, *L. diatas* sp. nov, *L. arathooni*, and *L. phyllofolia* described herein, *L. kejutan* sp. nov can most easily be

distinguished from all other remaining Sulawesi fanged frog species on the basis of geographic range, being solely restricted to the Southwest Peninsula south of the Tempe Depression.

Description of holotype.—An adult male, SVL 77.66 mm (FIGURE 3C, 4A); body strong and muscular; head gargantuan, superficially appearing as large or larger than the remaining length of the trunk (HW and HL 26.81% and 33.54% of SVL, respectively), and with enhanced conspicuity due to the prominence of paired postorbital hypertrophied muscles that bulge out from the cranium; moreover, head is slightly longer than wide, 125.10% of HW, with HW in life roughly equal to BW (HW/BW = 101.98%, in paratypes $\bar{x}$ = 92.90%); eye prominent, notably large and round with metallic gold iris and rhomboid pupil directly inferiorly bordered by a dark delta on the iris, roughly equal or less than interorbital distance (IOD/ED = 102.09%; in paratypes, $\bar{x}$ = 71.65%); eyes set wide, with IOD also being greater than the distance from the anterior margin of the eye to the nostril (IOD, 124.07% of END; in paratypes $\bar{x}$ = 144.85%); tympanum round and extremely reduced, 2.86 mm in diameter (on a frog with 77.66 mm SVL), and is at least 50% if not fully obscured beneath a prominent ridge-like supra-tympanal skin fold that initiates at the posterior margin of the eye, extending over and across the tympanum, terminating just above the arm; odontoids large and fierce, 4.28 mm in diameter and unshrouded by dermal tissue as they protrude upward from the mandible.

Arms muscular and indominable, with FAL roughly equal to UAL (FAL/UAL = 95.77%, %; in paratypes, $\bar{x}$ = 101.13%); fingers long, unwebbed, and bowed inward such that the thumbs point slightly backward toward the clavicles, with relative finger lengths III > IV > I > II (FIGURE 4A); hindlimbs long and especially brawny when allometrically scaled to smaller frogs in this assemblage, owing to a doubtlessly great leap-force and aquatic kick strength; feet and toes long, fully-webbed, with bulbous oviform subarticular tubercles and webbing formula, **I** 0–0^{+} **II** 0–0^{-} **III** 0^{-}–0 **IV** 1^{-}–0 **V** (FIGURE 4A). A distinct flap of skin (as if a continuation of the interdigital webbing) attaches peripherally to the toe pad at terminal phalange V about the 0^{+} position, runs the length of the foot, and terminates (exteriorly) at the proximal end of the metatarsal. An analogous flap is present peripherally on phalange 1, attaching at the 0^{+} position of the toe pad – running the length of the foot, and terminating at the 3 position of the inner metatarsal tubercle. Dorsal skin only weakly rugose and definitively un-warty, though most prominent rugosities occur on the flanks; dorsolateral dermal plicae prominent anteriorly just behind the hypertrophied jaw muscles, though tend to completely dissolve posterior to the forelimbs.

Coloration.—In life, *L. kejutan* sp. nov presents with a dark caramel dorsal, heavily mottled with irregular dark coffee-brown blotches (FIGURE 3C). Brown banding is prominent across the forearms, thighs, and tibia – and though appearing darker in life, the bands are still maintained in preservative (FIGURE 3C, 4A). Where other congeners might present with a dark mask that extends anteriorly from the snout and spans the tympanum, we have observed many individuals of *L. kejutan* sp. nov with a thin brown or black stripe that initiates at the snout, bisects the eye, and then subsequently outlines lower margin of the supra tympanal dermal fold (FIGURE 3). The venter (both in life and preservative) is slightly off-white from waist to chin with little to no mottling (FIGURE 4A). The brown pigmentation on the dorsal surface of the legs tends to bleed onto ventral surface, especially posteriorly, lending the under-leg slightly more brown than the rest of off-white venter (FIGURE 4A). Juvenile individuals may present with a dorsally olive-green appearance (FIGURE 3B).

Reproductive observations.—Though we did observe and record male *L. kejutan* sp. nov calling, we have not witnessed frogs in amplexus. Calling males appear to have weakly paired vocal sacs, but further field observations are needed to elucidate the reproductive behavior of sexes in this species. During our surveys, we collected three gravid females with roughly 40 eggs in their reproductive tracts, though we have not observed whether the frogs are terrestrial, oviparous, or aquatic egg layers.

Natural history.—In the large, fast-flowing streams that *L. kejutan* sp. nov inhabits, it is most certainly an apex predator across small vertebrates and large invertebrates (FIGURE 5A). We assume (as with other Ranoids) the animals are highly triggered by movement and will attempt to consume most anything that opportunistically comes within reach. During our collecting efforts, we've captured individuals only to find the legs of large *Polypedates leucomystax* protruding from the mouth, and assume the frogs readily prey on other proximate sympatric frogs in genus *Limnonectes*, *Hylarana*, or *Rhacophorus*, if given the opportunity. Two of the authors herein have even been bitten while reaching out to capture large individuals on Bawakaraeng! Though we do not yet know the full extent of diet composition for this species, we interestingly observed several animals with large freshwater crabs in their stomachs while preparing the specimens, thus adding to our assumption that *L. kejutan* sp. nov are both dietary generalists- and highly predatory.

Variation.—Though slight variation in color and rugosity are always probable, *L. kejutan* sp. nov are fairly unmistakable, being the largest fanged frog in their habitat. Due to the still-nebulous nature of potential contact zones with its low-elevation sister-species, *L. microtympanum* remains the only frog on Sulawesi's Southwest Peninsula with which proper field identification could be challenging.

Habitat Description.— In 2016, we collected the type series of *Limnonectes diatas* sp. nov while surveying the northeast face of Gunung Bawakaraeng, one of the twin peaks that compose the Gunung Lompobatang-Bawakaraeng massif. Most of our work was conducted between ~1520 m (where agricultural fields transition to natural forest) and the 2830 m summit of the mountain. Though stands of mature forest exist at higher elevations on the massif, the habitat of *L. diatas* sp. nov and *L. kejutan* sp. nov at the type locality included mostly mature, though apparently selectively-logged, closed canopy montane forest. In full, the aspect of the mountain we surveyed included a network of thin, rocky, low-flow, and shallow upland trickles at the highest elevations that graded into a vast network of headwater streams, offshoot channels, and plunge pools at mid- elevations, and finally leading to large, steep, fast-flowing streams and waterfalls at the base – the entire hydrological amalgam of which ultimately feeds into the Salo Tangka River complex at low elevations near Desa Malino. Our camp was established at 1700 m elevation along the upper reaches of the Salo Tangka (the Tangka River in the local Bugis language) immediately above a large (~100 m) waterfall. The main channel streams (preferred habitat for *L. kejutan* sp. nov) at this elevation ranged in width from 5-10 m, were typically less than 0.5 m in depth, and had clear, relatively fast-flowing water with many small to large boulders present (FIGURE 5A). The stream habitat at this elevation was heterogeneous, with the main channel characterized by numerous plunge pools between 0.75 and 1.5 m deep (FIGURE 5B). The main channel stream supported a vast network of thin, rocky, low-flow, offshoot channels and shallow trickles that comprised the preferred habitat of *L. diatas* sp. nov (FIGURE 5C). The Salo Tangka ultimately flows eastward into the Gulf of Bone at the town of Sinjai. During our surveys, we observed the highest densities of *L. diatas* sp. nov along mid-elevation

stream networks between 1670—1720 m, but we focused our surveys out of our main field camp to this elevational band because of the abundance of appropriate habitat for both species (although it was nevertheless challenging to collect). That said, we believe both these species' habitats likely extend substantially higher on the mountain and could potentially reach 2400 m or more given that the headwaters of the Salo Tangka extend to at least this elevation. We surveyed higher elevation sites between 2400—2500 m but did not encounter the species above 1719 m primarily because our route did not intersect with appropriate stream habitat. Species turnover from *L. arathooni* at lower elevations to *L. diatas* sp. nov in the uplands occurred between 1561 m (the highest elevation at which we collected *L. arathooni*) and 1672 m (the lowest elevation at which we collected *L. diatas* sp. nov). Species turnover from *L. microtympanum* at lower elevations to *L. kejutan* sp. nov in the uplands occurred between 1241 m (the highest elevation we collected *L. microtympanum*) and 1485 m (the lowest elevation at which we collected *L. kejutan* sp. nov). These collecting sites were separated by large waterfall, but there was otherwise no obvious change in the surrounding habitat. Both new species are reliably found in sympatry with one another. We observed that *L. diatas* sp. nov tends to prefer steep-banked, slower moving side channels and spray zones emanating from larger headwater streams, as the *L. kejutan* sp. nov is abundant along the main channel and may be a primary predator on *L. diatas* sp. nov (FIGURE 5A). We commonly heard males of *L. diatas* sp. nov calling before they were visually detected (even during the day) and quite often would find them in small holes in the conglomerate mud and gravel substrate, perched at the opening of mossy rock crevices, or deep within tangled networks of sticks, roots, leaf-litter, and other debris along the side of the low-flow offshoot channels (FIGURE 2C, 5D). *L. kejutan* sp. nov was conspicuous and preferred higher flow rate zones – often found either sitting at the base of large mid-stream boulders just above the waterline, or on wet mossy banks at the edge of large plunge pools (FIGURE 5B).

Morphometric Comparisons.—For both cryptic species pairs, we report redundant statistically significant differences in limb and body measurements across both orthogonal and non-transformed frequentist scales. The sample mean ($\bar{x}$), standard deviation (SD), and range of morphological characters for both novel species, *L. diatas* sp. nov and *L kejutan* sp. nov, are given in Table 1.

Resultant eigenvalues from the orthogonal functions generated using 19 morphological characters for *L. diatas* sp. nov and *L. arathooni* explain 70.39% of the model variance within PC1, 9.60% of the variance within PC2, and 4.70% of the variance within PC3 – with the first three PCs accounting for 84.81% of overall model variance. Clustering between the two species is shown in FIGURE 6A. The 20 character morphometric data set for both species comprised of 11 L. diatas sp. nov and 9 *L. arathooni*; ergo, a full MANOVA necessitates more degrees of freedom (e.g., could be ameliorated by increasing our samples sizes of each species). As such, we opted to run separate ANOVAs on the PC scores for each of the first three dimensions. For PC1, there were highly significant differences between the two species ($\alpha < 0.001$; $P = 2.723^{e-09}$). Model results were not significant at any level of alpha for either PC2 or PC3 ($\alpha < 0.001$; $P = 0.3855$, and 0.676, respectively), thus, we chose to extract the variance components of PC1 for downstream tests on the non-orthogonally transformed data. Contributing variables to the overall variance in PC1 were as follows: THL, HW, TL, LAL, FL, FAL, SVL, HNDL, END, BW. Mann-Whitney U tests for morphometric differences between the two species were highly significant in all cases (TABLE 2).

For our comparison of *L. microtympanum* and *L. kejutan* sp. nov PCA eigenvalues explained 65.67% of the model variance within PC1, 9.64% of the variance within PC2, and 6.55% of the variance within PC3 – with the first three PCs accounting for 81.87% of overall model variance. The 19-character morphometric data set for both species comprised of 21 *L. kejutan* sp. nov and 25 *L. arathooni*; thus, degrees of freedom were sufficient for MANOVA. Results were highly significant at any level of confidence with ($\alpha < 0.001$; $P = 4.225e^{-05}$**).** For downstream interpretive purposes, we subsequently extracted the variance components owed to each morphological character from the first PC that contributed at least 5% of the cumulative variance within that dimension (effectively signaling which characters, if any, were most relevant to morphological divergence). Ten characters met this criterion, including: HW, SVL, THL, IOD, HNDL, FAL, FL, UAL, TL, and LAL. Nevertheless, as we collected enough frogs to meet degree of freedom requirements for a MANOVA, we opted to perform Mann-Whitney U tests on all 19 characters measured. Results are summarized in TABLE 1; however, in brief, we report significant differences in 14 of the 19 characters. There were no significant differences between the two species owed to: TL, SNL, TD, ETD, or OPR.

Molecular Comparisons.—Results of our maximum likelihood phylogenetic estimations are reported in FIGURE 6E. Internal node bootstrap support values were ubiquitously 99-100%, with clear distinctions between low and high elevation sister-species pairs. All individuals among the previously presumed *L. arathooni*- and *L. microtympanum* assemblages captured at 1580 m or above are herein demarcated as *L. diatas* sp. nov (*L. arathooni* high-elevation analog) and *L. kejutan* sp. nov (*L. microtympanum* high-elevation analog). For the complete set of exonic loci, uncorrected pairwise genetic distance for the *L. arathooni—L.diatas* sp. nov assemblage = 6.7%. For the *L. microtympanum—L. kejutan* sp. nov assemblage, uncorrected pairwise genetic distance = 11.6%. Results from STRUCTURE HARVESTER analyses corroborated our phylogenetic estimates, revealing the most likely number of populations in each comparative case to be K = 2 populations. Results plots of STRUCTURE analyses for K = 2 populations in both cases are depicted in FIGURE 6B, C.

Acoustic Comparisons.— Our opportunistic surveys resulted in the collection of two calls from n = 2 *L. arathooni*. eight calls from n = 3 *L. diatas* sp. nov. One-second spectrogram and waveform samples of advertisement calls from each species are shown in (FIGURE 7C, D). L. *arathooni* calls differed from *L. diatas* sp. nov calls based on mean note number (NN), call duration (CD), and pulse rate (PR), dominant frequency (DF), though not enough calls were collected between the two species to satisfy sample size requirements for statistical significance testing. In summary, *L. arathooni* uttered more notes ([*L. arathooni*] $\bar{x}$ NN = 6.5 notes SD = 0.707; [*L. diatas sp. nov*] $\bar{x}$ NN = 2.6 notes, SD = 0.518), had a shorter call duration ([*L. arathooni*] $\bar{x}$ CD = 0.905 sec, SD = 0.103; [*L. diatas* sp. nov] CD = 1.452 sec, SD = 1.322), had a higher pulse rate ([*L. arathooni*] $\bar{x}$ PR = 6.072 notes/sec, SD = 0.089; [*L. diatas* sp. nov] $\bar{x}$ PR = 2.431 notes/sec, SD = 2.292), and had a slightly higher dominant frequency ([*L. arathooni*] $\bar{x}$ DF = 2.422 kHz, SD = 0.062; [*L. diatas* sp. nov] $\bar{x}$ DF = 2.351 kHz, SD = 0.349) in their advertisement call compared to their high elevation congener.

We were ultimately able to record three calls from n = 1 *L. microtympanum* and five calls from n = 1 *L. kejutan* sp. nov. One-second spectrogram and waveform samples of advertisement calls from each species are shown in (FIGURE 7A, B). *L. microtympanum* calls differed from *L. kejutan* sp. nov calls based on mean note number (NN), call duration (CD), and pulse rate (PR), dominant frequency (DF), though not enough calls were collected between the two species to

satisfy sample size requirements for statistical significance testing. For the sister-species pair, the low-elevation analog - *L. microtympanum* uttered more notes ([*L. microtympanum*] $\bar{x}$ NN = 29.67 notes, SD = 3.79; [*L. kejutan* sp. nov] $\bar{x}$ NN = 2.67 notes, SD = 1.516), had a longer call duration ([*L. microtympanum*] $\bar{x}$ CD = 3.040 sec, SD = 0.373; [*L. kejutan* sp. nov] CD = 0.580 sec, SD = 0.898), had a higher pulse rate ([*L. microtympanum*] $\bar{x}$ PR = 9.753 notes/sec, SD = 0.049; [*L. kejutan* sp. nov] $\bar{x}$ PR = 4.622 notes/sec, SD = 0.840), and had a slightly lower dominant frequency ([*L. microtympanum*] $\bar{x}$ DF = 0.944 kHz, SD = 0.053 ; [*L. kejutan* sp. nov] $\bar{x}$ DF = 1.090 kHz, SD = 0.383) in their advertisement call compared to their high elevation congener.

DISCUSSION

The discovery of *L. diatas* sp. nov. and *L. kejutan* sp. nov exemplify the inherent difficulty in detecting cryptic species in a biodiversity hotspot. In the field, we noticed slight body size differences between frogs at low and high elevation sites that were presumed to be the same species. That said, fanged frogs across the island quite often exhibit morphologically similar phenotypes, while single-species assemblages can be composed of variable color morphs, and further-, may exhibit sexual dimorphism in overall body size. This amalgam of factors precipitating in morphological crypsis and taxonomic uncertainly has been widely reported across Southeast Asian anurans (Brown et al 2010; Sheridan and Stuart 2018; Stuart et al. 2006; Wogan et al. 2016; Kotaki et al. 2010). Moreover, morphological character divergence tends to evolve more slowly in cryptic frog species, especially when compared to divergence owed to barriers to gene flow, or reproductively isolating phenotypes (Angulo and Icochea 2010; Funk et al. 2012; Padial et al. 2008; Sheridan and Stuart 2018; Stuart et al. 2006). Though admittedly difficult to detect in the field, we found that *L. diatas* sp. nov morphologically differed from its low elevation sister species in nearly all limb length measurements, as well as SVL, BW, and END. We also found congruent results between *L. kejutan* sp. nov and *L. microtympanum*, with most of the statistically significant variation in our models being owed to limb-length characters (THL, HNDL, FAL, FL, UAL, TL, LAL), as well as HW, SVL, IOD. Interestingly, other prominent studies of elevational speciation in frogs (e.g., *Pseudacris maculata*: see Funk et al. 2016) report that high-elevation species analogs exhibited larger overall body size than their low-elevation counterparts (in seeming agreement with Bergmann's clines); however, *L. diatas* sp. nov. and *L. kejutan* sp. nov. demonstrate the opposite pattern – being notably smaller in body size compared to their lowland obligate counterparts. In fact, we've observed that across nearly all widely distributed montane frog genera on Sulawesi, high elevation obligates are invariably miniaturized with increasing elevation (e.g., *Rhacophorus*, *Oreophryne*, *Hylarana*, and *Limnonectes),* lending credence to purported findings that broad rules predicting increases in body size with decreasing temperature and increasing altitude should not be broadly assumed for amphibians (Adams et al. 2008; Cvetkovic et al. 2009). Taken together, our morphological investigations highlight the importance of high-density sampling and careful consideration regarding statistical testing when attempting to elucidate cryptic species. *In situ* field observations or single-method statistical analyses on a "presumed species" may hinder the probability of detecting hidden diversity (Patel et al. 2021; Ramesh et al. 2020; Sheridan and Stuart 2018; Scherz et al. 2019; Stuart et al. 2006)

Here, we emphasize integrating exploratory Sanger screening-, NGS genomic-, and demographic analyses when elucidating instances of cryptic speciation or attempting to demarcate known species complexes (Chan et al. 2022; Funk et al. 2016; Inger and Stuart 2010; Jaynes et al. 2021; Sheridan and Stuart 2018; Stuart et al. 2006). Preliminary Sanger sequencing of mitochondrial markers offers a low-cost, first-principles approach to detect cryptic species and facilitates subsampling of large collection series for fine-scale NGS screening. Our phylogenetic estimates of fanged frog mitogenomes indeed indicated support for both our field-based anecdotal hypotheses-, as well as our Sanger sequence-based assumptions that we inadvertently collected separate high-low elevation species analogs. Results from our structure analyses using ~7000 loci and K = 2 populations further indicate the need for the taxonomic split. We found no evidence of admixture among populations: a non-intuitive result - given that we could find no discernable differences in habitat type or quality that would implicate a tangible physical barrier capable of maintaining allopatry-, isolation by distance, nor any obvious environmental factors that could directly restrict gene flow among respective sister-species pairs. Moreover, in both cases, species turnover at the type locality for *L. diatas* sp. nov. and *L. kejutan* sp. nov. occurred within a remarkably narrow (110-120 m) elevation band, and we expect that in other localities the elevation of a contact zone or demarcation line of species turnover may be mutable. In short, without a physical barrier to gene flow, and given our findings of no admixture, the STRUCTURE results corroborate our phylogenomic and distance estimates insofar as the respective sister-species are certainly divergent entities.

Acoustic analyses revealed interesting manifestations of reproductive phenotypic divergence, in that, characters between the high-low elevation analogs reliably differed, though incongruently relative to sister species pairs. Notably, the only common threads between both cases of phenotypic call differentiation were reductions in note number and pulse rate for both *L. diatas* sp. nov. and *L. kejutan* sp. nov. compared to the lowland frogs. Mechanistically, lower note numbers and pulse rates are often correlated with body temperature, as physiological expenditures are thus more energetically costly for frogs at higher altitudes than their lowland counterparts (Gergus et al. 1997; Gergus et al. 2004; Padial et al. 2008). Although some studies purport that larger-bodied frogs usually have a higher dominant frequency and longer call durations (e.g., Padial et al. 2008; Sullivan et al. 2000; Marquez et al. 1995) we report no discernable patterns for both species pairs with respect to call duration and dominant frequency – though they did differ in each case. Insofar as no discernable physical barrier of isolation between high-low elevation analogs-, nor notable turnover in habitat type or availability could be identified, further acoustic investigations with denser sampling are needed to clarify the yet-open question of mate recognition and reproductive biolological mechanistics involved with both *L. diatas* sp. nov and *L. kejutan* sp. nov lineage divergence, as well as the maintenance of non-introgression. Pulse rates are pivotal for anuran mate recognition and have been suggested as a key mechanism for population differentiation and speciation among assemblages with spatially broad range extents (Angulo and Icochea 2010; Gergus et al. 1997; Guerra and Ron 2008; Padial et al. 2008; Sullivan et al. 2000). Moreover, several studies report advertisement call characters as plastic and variable across populations, indicating that bioacoustics are playing a dominant role in the initiation of ecological and/or adaptive speciation through mate recognition when compared to the evolution of morpho-types (Angulo and Icochea 2010; Funk et al. 2012; Gergus et al. 1997; Padial et al. 2008; Stuart et al. 2006). This key insight assuredly underscores the common difficultly faced by investigators in detecting cryptic species; ergo, we recommend that

robust call analyses be routinely implemented in studies aiming to taxonomically diagnose cryptic species or the presence of species complexes.

In summary, with so few Sulawesi fanged frogs being formally described in the literature, correct species identification in the field has proven quite challenging. Herein, we've shown two remarkable cases of cryptic speciation, in that two high-low sister species pairs demonstrated turnover at a notably narrow-, ecologically homogenous contact zone. *In situ*, we've tended to rely on our experiential knowledge for species identification, considering tentative geographic ranges and observational assessments of morphology including flash coloration, overall body size, habitat preference, and degree of interdigital webbing. This study highlights the benefits of integrative NGS molecular methods – coupled with morphometric and acoustic analyses. Given aforementioned challenges regarding the detection of crypsis and the hypothesized adaptive radiation of Sulawesi *Limnonectes* (see Setiadi et al. 2011), we expect that our efforts to resolve the systematics and taxonomy of this assemblage will undoubtedly result in the further discovery of fascinating cryptic species, all of whom may exhibit any number of extraordinary life history characteristics, reproductive modes, eco-morphologies, or population dynamics (Evans et al. 2003; Frederick et al. 2022; Iskandar et al. 2014).

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FIGURES AND TABLES

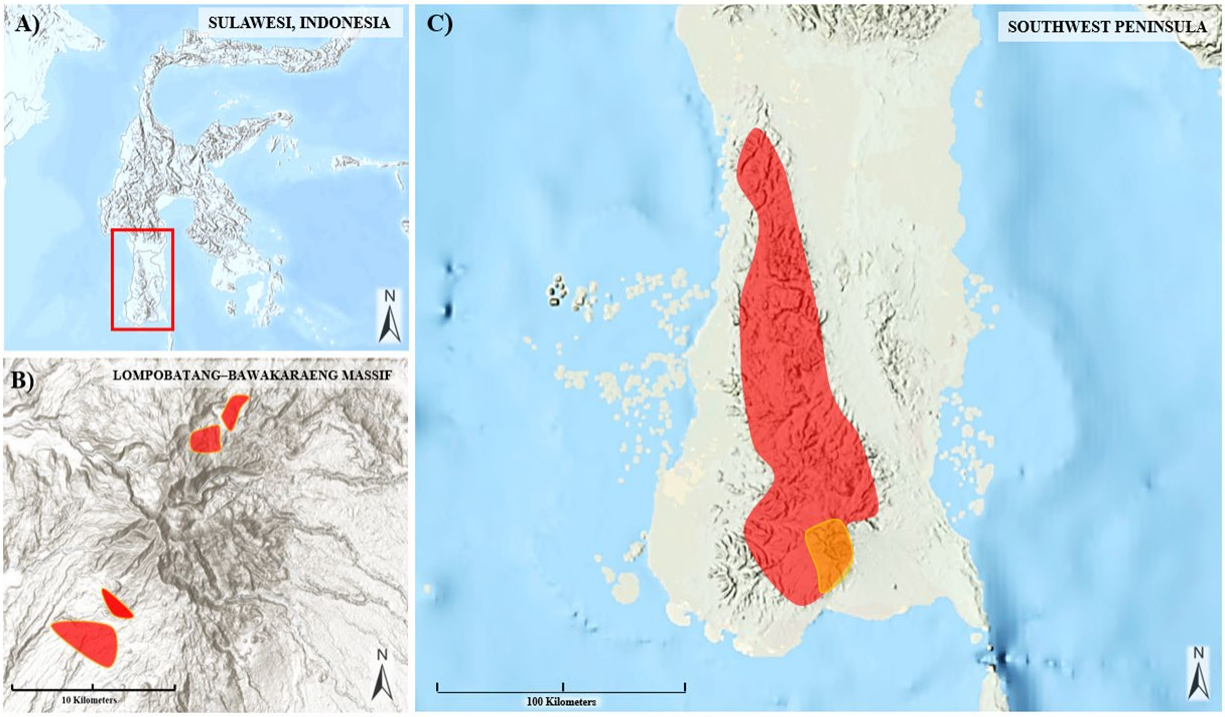

Figure 1—Sulawesi Southwest Peninsula regional-, type-, and predicted range localities for the focal *Limnonectes* spp. described in this study. Respective panels depict: (A) the entirety of our study area, (B) the type locality for *L. diatas* and *L. kejutan* sp. nov (the Lompobatang-Bawakaeaeng Massif), and (C) the assumed range (Panel C: region in red encompassing Bantimurung National Park) of all four focal species: *L. arathooni*, *L. microtympanum, L. diatas* sp. nov, and *L. kejutan* sp. nov. The orange region in Panel C encompasses both the Lompobatang-Bawakaeaeng Massif and type localities for *L. diatas* sp. nov and *L. kejutan* sp. nov. Within the spatial extent both orange and red regions in Panel C, we assume *L. arathooni* and *L. microtympanum* to be present below 1600 m elevation, while *L. diatas* sp. nov and *L. kejutan* sp. nov.

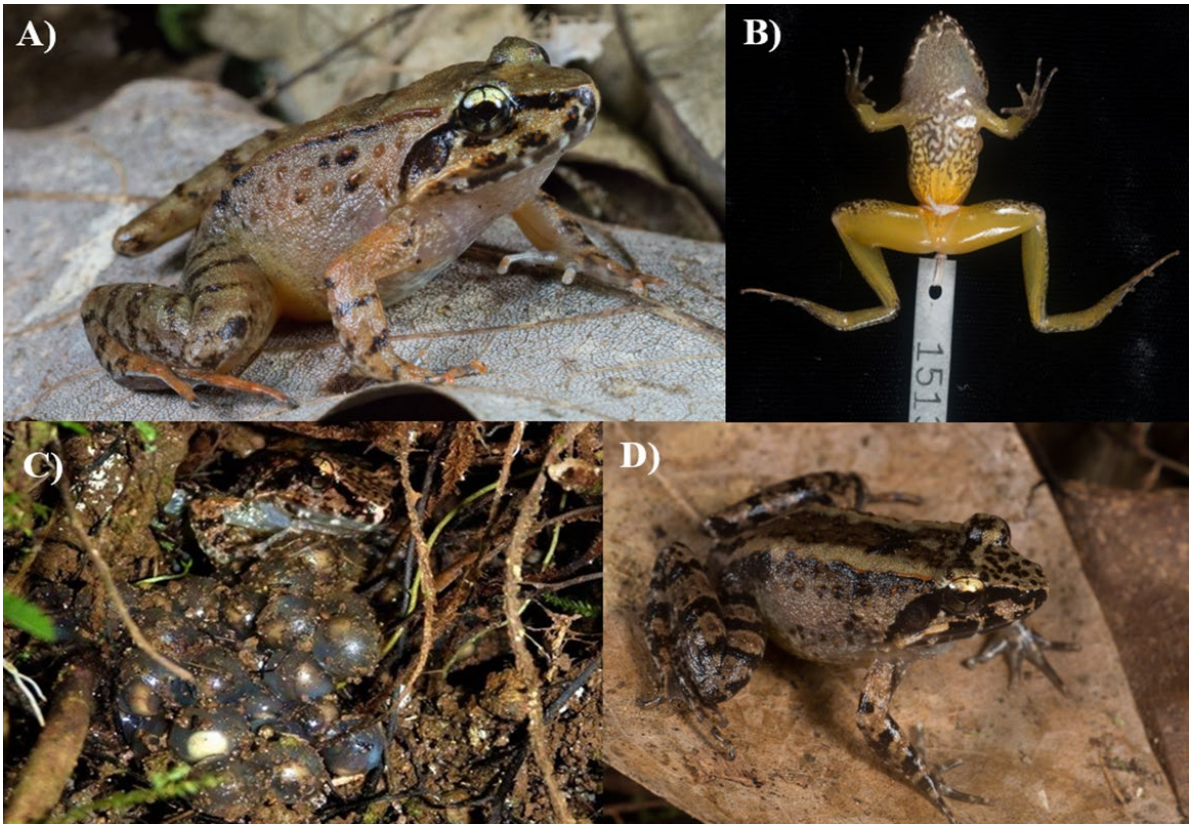

Figure 2—Images of *Limnonectes diatas* sp. nov. in life. Panels (A), (B): JAM 15135 [Holotype], adult male, captured 30 Oct 2016 on the north face of G. Bawakaraeng: 1699m elevation. Panel (B): diagnostic yellow ventral flash coloration in this species. Panel (C), (D): JAM 15046, adult male, captured 21 Oct. 2016 while guarding two clutches of eggs 1m from the edge of a shallow, low-flow stream channel on the north face G. Bawakaraeng (see Fig. 5C for detail): 1717m elevation. Panel (C) depicts characteristic terrestrial egg deposition and male egg guarding behavior; Panel (D) depicts the barred morph of the bi-phasic color polymorphism exhibited by both male and female exemplars of this species (compare with Panel (A)).

Figure 3—Images of *Limnonectes kejutan* sp. nov. in life. Panel (A): JAM 14908, adult male, captured 13 Oct 2016 on the north face of G. Bawakaraeng: 1713m elevation. (B): JAM 15135, juvenile male, captured 13 Oct 2016 on the north face of G. Bawakaraeng: 1711m elevation. Panel (C) [Holotype]: JAM 14899, adult male, captured 13 Oct. 2016 on the north face of G. Bawakaraeng: 1738 m elevation.

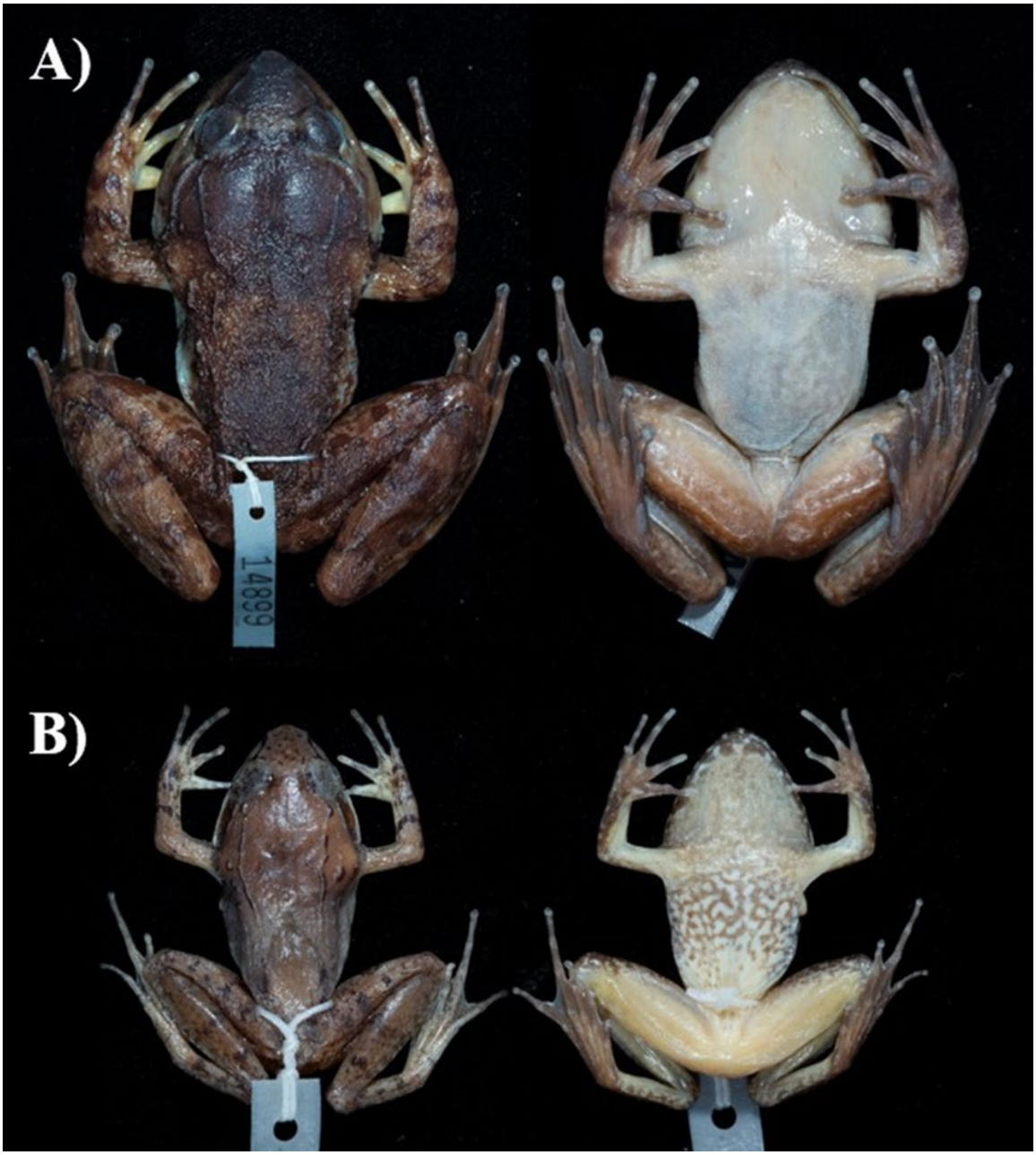

Figure 4—Dorsal and ventral views of (A) *Limnonectes kejutan* sp. nov and (B) *Limnonectes diatas* sp. nov Holotypes (JAM 14899; JAM 15135) depicting diagnostic morphological characters, hand and foot profiles, and specimen coloration in preservative.

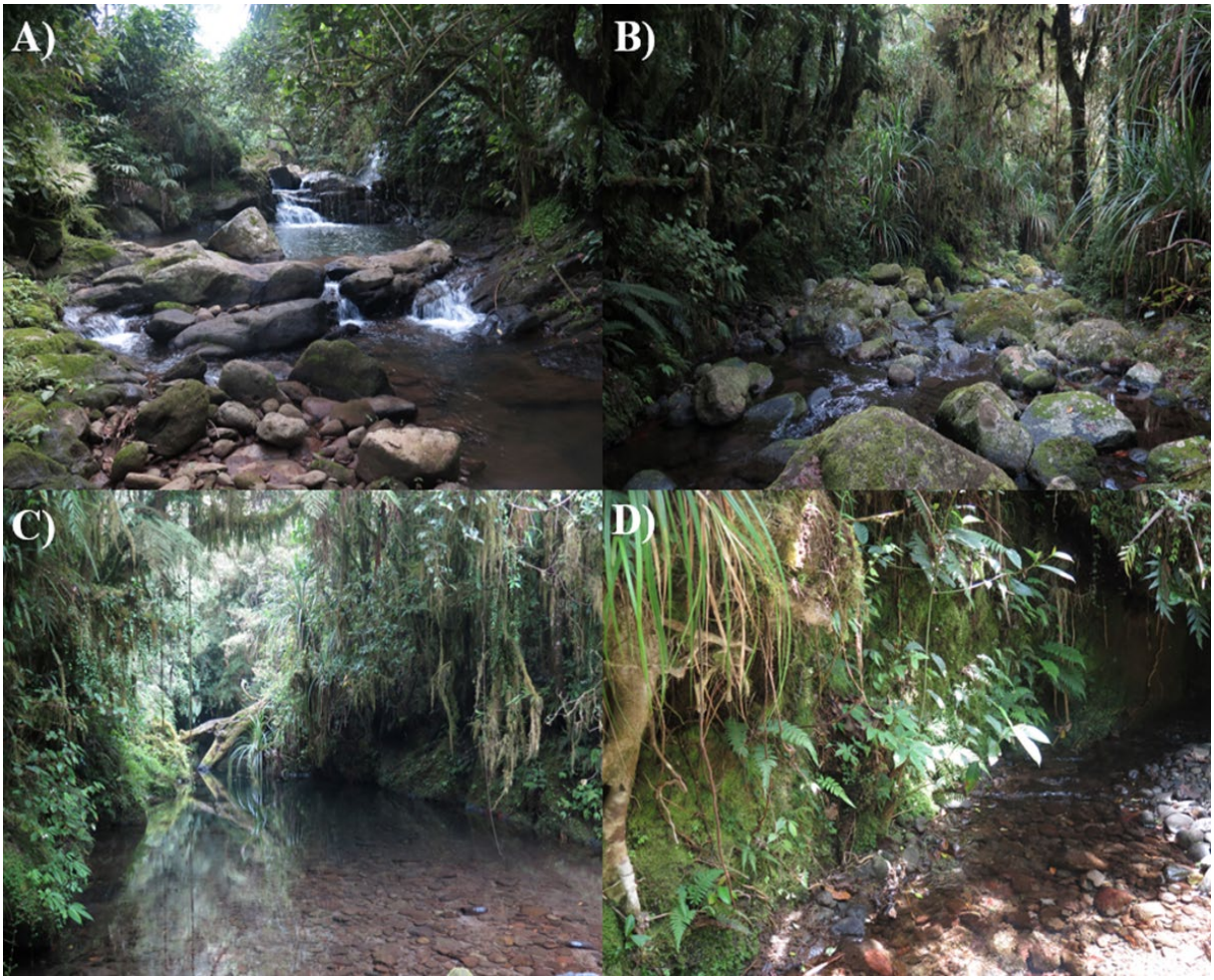

Figure 5—Images of typical *Limnonectes* habitat at the type locality on the north face of Gunung Bawakaraeng. Panel (A) depicts both high-flow hydrological- and adjacent forest structure within the narrow elevational band (~1300 m) demarcating species turnover among *L. arathooni* —*L. microtympanum* at the base of the mountain, and *L. diatas* sp. nov—*L. kejutan* sp. nov. in the uplands. Panels (B, C) depict the typical stream hydrology and adjacent forest structure preferred by *L. kejutan* at ~1700m [(B) high-flow cascades; (C) broad plunge pools]. Panel (D) depicts a shallow side-channel adjacent to the main streams in the headwater network with steep mossy banks [~1700 m]: the preferred breeding / nesting habitat of *L. diatas* sp. nov.

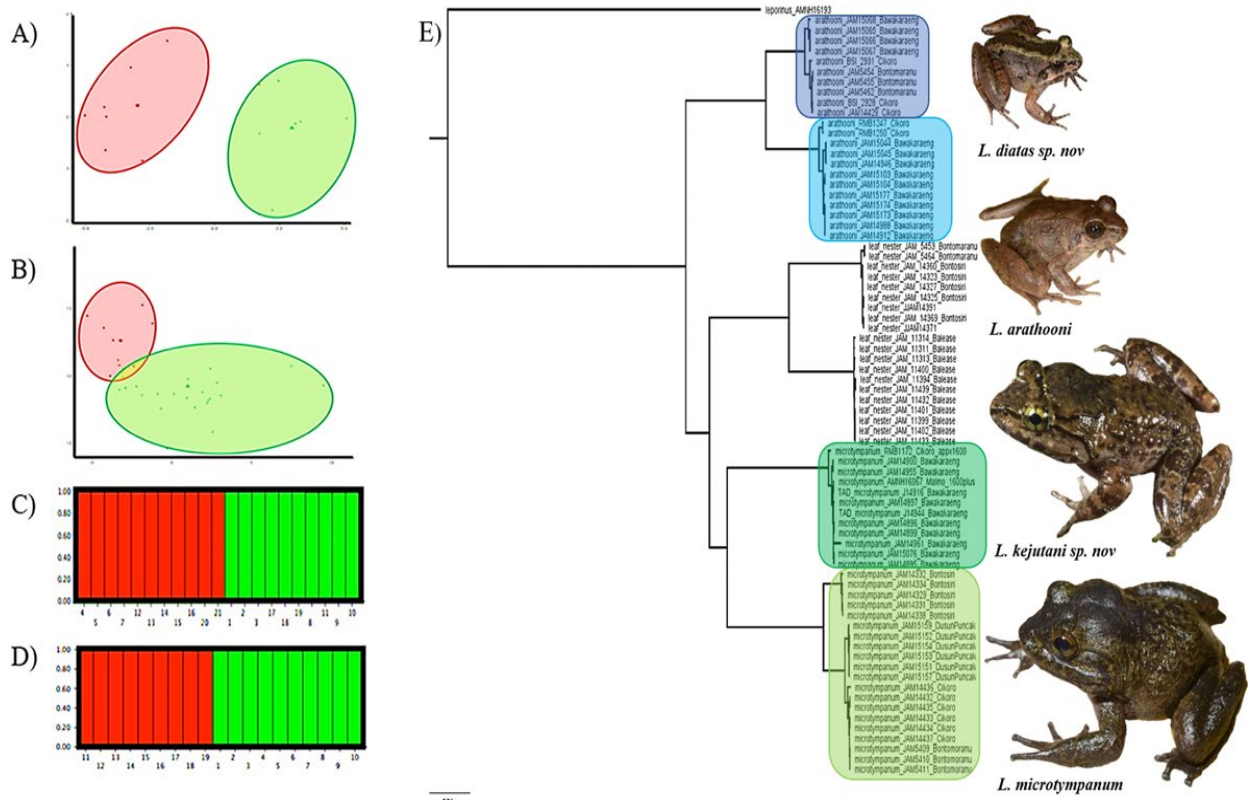

Figure 6—Orthogonal morphometric (A; B), demographic (C; D), and phylogenomic (E) comparisons between previously described lowland (*L. arathooni* and *L. microtympanum*) and upland (*L. diatas* sp. nov. and *L. kejutan* sp. nov.) fanged frog sister species on Sulawesi's Southwest Peninsula. Panels (A; *L. arathooni-diatas*) and (B; *L. microtympanum-kejutan*) depict orthoganal clustering between sister species based on 20 morphological characters. Panels (C; *L. arathooni-diatas*) and (D; *L. microtympanum-kejutan*) show STRUCTURE plot results for K=2 populations based on SNP data from ~5,800 SNPs, and a 1M generation run-time. Panel (E) summarizes our maximum liklihood phylogenomic estimate for both sister species pairs based on full mitogenomes (~18,300bp alignment). All nodes had ≥ 99% bootstrap support values. Inset images depict the focal species compared from Lompobatang-Bawakaraeng Massif, Sulawesi.

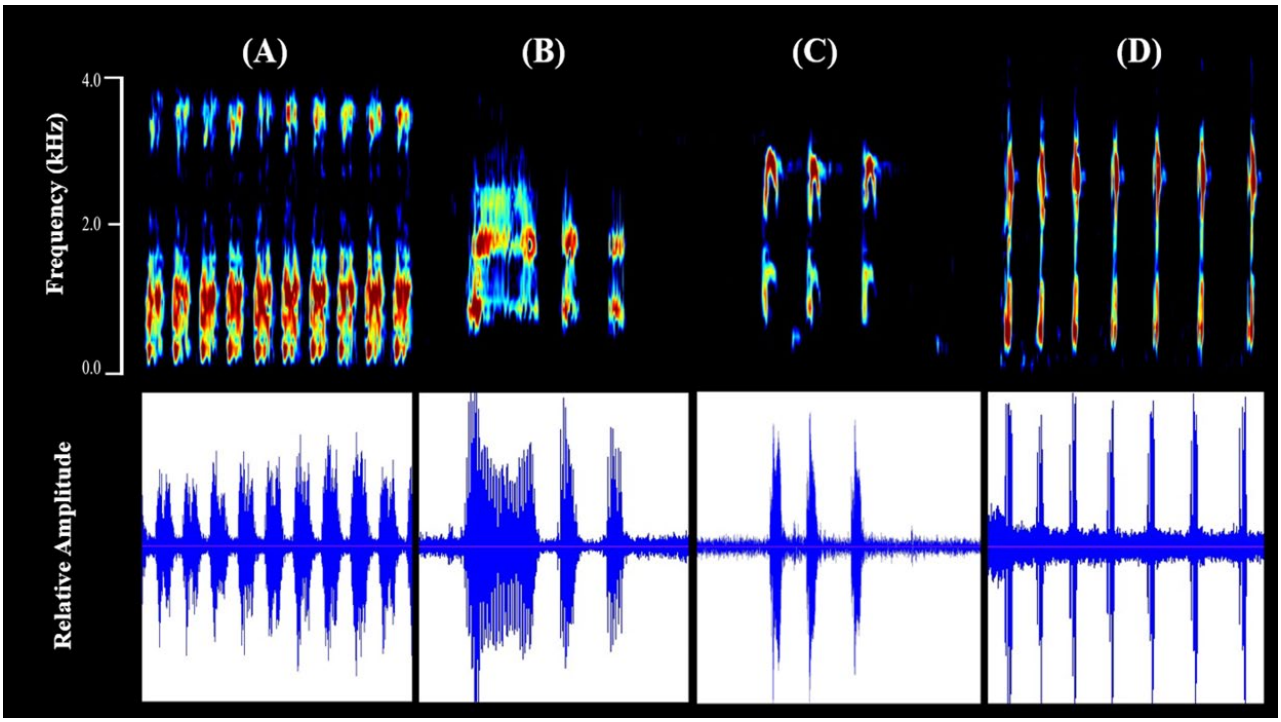

Figure 7—Comparative one second exemplar pairs of spectrograms (kilohertz vs. time) and waveform oscillograms (relative amplitude vs. time) calculated for: (A) *L. microtympanum*, (B) *L. kejutani* sp. nov., (C) *L. diatas* sp. nov., and (D) *L. arathooni.* Spectrograms and waveforms were ubiquitously calculated using a Fast Fourier transformation value of 512.

Morphological Characters	***L. diatas* sp. nov** *[n=14 Males]*			***L. kejutani* sp. nov** *[n=21 Males]*		
	Range	$\bar{x}$	St. Dev.	Range	$\bar{x}$	St. Dev.
Head length (HL)	14.51—9.91	**11.96**	±1.41	25.62—33.54	**29.37**	±2.4
Head width (HW)	11.55—16.92	**13.08**	±1.77	20.85—33.22	**27.95**	±3.5
Snout-Vent Length (SVL)	26.20—44.30	**31.9**	±4.93	60.19—77.66	**67.68**	±4.36
Tibia Length (TL)	14.71—24.35	**17.47**	±3.05	33.66—42.03	**37.33**	±2.43
Interorbital Distance (IOD)	2.44—4.58	**3.34**	±0.53	5.05—7.08	**6.14**	±0.52
Eye Diameter (ED)	2.75—5.22	**3.82**	±0.73	7.69—9.40	**5.77**	±0.44
Internarial Distance (IN)	2.85—4.52	**3.36**	±0.43	4.99—6.48	**6.05**	±0.43
Eye-Nostril Distance (EN)	2.25—3.55	**2.74**	±0.43	4.68—6.78	**5.77**	±0.59
Foot Length (FL)	14.44—17.72	**16.96**	±2.38	34.47—40.82	**37.78**	±2.02
Tympanum Diameter (TD)	2.13—2.78	**2.43**	±0.23	2.58—4.49	**3.41**	±0.46
Thigh Length (THL)	14.81—24.44	**17.57**	±2.86	33.79—41.05	**34.47**	±1.99
Snout Length (SL)	3.04—4.86	**3.98**	±0.67	8.26—11.00	**9.94**	±0.72
Hand Length (HAL)	7.84—11.72	**9.03**	±1.24	17.58—22.39	**13.38**	±1.16
Forearm Length (FLL)	5.50—9.15	**6.25**	±1.04	12.07—14.86	**31.19**	±0.91
Eye-Tympanum Distance (ETD)	1.21—2.10	**1.54**	±0.27	4.07—10.98	**7.00**	±2.04
Snout-Nostril Length (NS)	0.97—2.22	**1.64**	±0.5	2.73—6.03	**4.81**	±1
Upper Arm Length (UAL)	5.09—8.72	**6.47**	±0.96	11.73—14.98	**13.38**	±0.88
Lower Arm Length (LAL)	12.31—18.21	**15.06**	±1.85	28.02—34.75	**31.19**	±1.69
Body Width (BW)	8.77—18.88	**11.98**	±2.67	22.19—34.34	**29.42**	±3.37
Odontoid Process Length (OPL)	0.98—2.08	**1.35**	±0.27	2.19—4.52	**3.49**	±0.78

Table 1— Non-transformed ranges, means, and standard deviations of body size and limb length character measurements (in mm) for adult male *L. diatas* sp. nov. and *L. kejutani* sp. nov. samples used in this study (including the holotype).

	L. microtympanum ***L. kejutani* sp. nov**			***L. arathooni*** ***L. diatas* sp. nov**		
Character	[MW-U] *P*	Sig.	PC1 Var.	[MW-U] *P*	Sig.	PC1 Var.
HW	$1.30\ e^{-04}$	*	†	$1.19\ e^{-05}$	*	†
SVL	$7.51\ e^{-07}$	*	†	$1.19\ e^{-05}$	*	†
THL	$2.02\ e^{-04}$	*	†	$1.19\ e^{-05}$	*	†
IOD	$2.86\ e^{-04}$	*	†	$1.40\ e^{-03}$	*	N/A
HNDL	$1.40\ e^{-05}$	*	†	$1.19\ e^{-05}$	*	†
FAL	$5.70\ e^{-05}$	*	†	$1.95\ e^{-04}$	*	†
FL	$1.68\ e^{-04}$	*	†	$1.19\ e^{-05}$	*	†
UAL	$1.42\ e^{-04}$	*	†	$1.96\ e^{-04}$	*	N/A
TL	$6.36\ e^{-02}$	N/A	†	$1.19\ e^{-05}$	*	†
LAL	$1.42\ e^{-04}$	*	†	$1.96\ e^{-04}$	*	†
SL	$1.20\ e^{-05}$	*	N/A	$1.21\ e^{-02}$	N/A	N/A
SNL	$1.89\ e^{-01}$	N/A	N/A	$2.61\ e^{-01}$	N/A	N/A
TD	$1.10\ e^{-01}$	N/A	N/A	$3.80\ e^{-03}$	*	N/A
BW	$4.58\ e^{-05}$	*	N/A	$4.76\ e^{-05}$	*	†
ETD	$3.94\ e^{-02}$	N/A	N/A	$1.60\ e^{-01}$	N/A	N/A
HL	$8.94\ e^{-03}$	*	N/A	$7.98\ e^{-04}$	*	N/A
END	$5.83\ e^{-03}$	*	N/A	$6.23\ e^{-04}$	*	†
OPR	$6.24\ e^{-02}$	N/A	N/A	$5.45\ e^{-03}$	*	N/A
ED	$5.83\ e^{-03}$	*	N/A	$3.00\ e^{-02}$	N/A	N/A

Table 2—Summary results of comparative morphometric analyses between low and high elevation analog species based on raw measurements from 20 characters. *P* values ($\alpha \geq 0.001$) from Mann-Whitney U tests on individual characters are given, with adjacent columns noting statistical significance (*) and variance component results (†), indicating whether a particular character contributed at least 5% of overall model variance to the first PC in our empirical orthogonal functions.

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CHAPTER 3

Differential Dehydration in a Niche-Partitioned Frog Radiation

ABSTRACT

Amphibians are widely known to lose water to the environment through cutaneous evaporative water loss; thus, despite their global ecological success, their life histories, behavior, and distributions are inevitably constrained by dehydration risk. Both adaptive and plastic responses to dehydration have been described, but such strategies are often lineage-specific and represent a unique eco-physiological interplay among thermal regimes, habitat selection, activity states, reproductive energetic expenditure, and metabolic requirements. Recently, integrative mechanistic biophysical modeling has been used to profile species distributions, conservation risks, differential habitat use, and niche partitioning; however, precise metrics of species-specific water loss potential and mass transfer are often excluded or derived by proxy. In order to facilitate downstream ecological niche modeling efforts in a radiation of equatorial stream frogs, we aimed to collect and compare water loss metrics across the Sulawesi *Limnonectes* fanged frog assemblage. Though iterative water loss trials conducted in the field, we report statistically significant differences in water loss and mass across different niche obligate species – while also discovering biologically relevant behavioral responses to dehydrating environmental conditions. Our findings highlight the dynamic strategies and trade-offs that sympatric frogs employ to maintain both water balance and novel ecological success within tropical stream communities.

INTRODUCTION

"[Amphibians are-] some of the most susceptible lineages to environmental variation…and are often regarded as the most threatened vertebrates worldwide. The claim of "high amphibian sensitivity", which is often invoked in conservation-oriented research, underestimates the great diversity in resilience to environmental change that characterizes this group."

(Bovo et al. 2018)

Ever adaptable, anurans are distributed across most of the Earth's terrestrial habitats outside of Antarctica and demonstrate an impressive array of eco-morphological and eco-physiological phenotypes. Because of this propensity toward ecological specialization, frogs have also colonized every major habitat type from deserts to rainforests and constitute nearly 90% of all amphibians (AmphibiaWeb 2016). The phenotypic variation and adaptations that have facilitated their global ecological success are nevertheless checked by one primary environmental variable: they are highly constrained by the need to maintain water balance (Lillywhite 2006). A frog's relationship to water balance is multi-faceted, owing in part to a complex life cycle wherein eggs and larvae must remain hydrated to proceed through

ontogenetic development into their adult forms. Moreover, frogs have a permeable integument and absorb the majority of their free water through their skin (Dodd 2010).

Water exchange and osmotic regulation are mediated by anuran integument due to the thin structure of the skin. As in other vertebrate organisms, the integument is multi-layered – though in frogs, only two epidermal layers of the stratum corneum are present and they lack the lipid density, lamellar bodies, and concentration of keratinized cells that are abundant in all other vertebrates (Dodd 2010, Lillywhite 2006, Pough 2016). This reduced structure is necessary for cutaneous respiration and osmotic regulation; however, it also puts the animals at substantial risk of dehydration and renders frogs especially susceptible to water loss (Bartelt 2000, Child et al. 2008; Dodd 2010; Lillywhite and Mittal 1999, Lillywhite and Navas 2006). Indeed, outside of water evacuated from in the bladder, frogs and all other amphibians primarily lose water freely to the environment through cutaneous water loss (Duellman and Trueb 1994, Preest et al. 1992; Toledo and Jared 1993).

Given this broad susceptibility to desiccation, hydric state strongly shapes anuran life histories and behaviors by influencing their physiological performance. In neotropical bufonids, Titon et al. (2010) found that desiccation sensitivity differentially affected locomotor performance in three species of *Rhinella*, and in turn, coincided with shifts in activity states across a range of temperatures – pointing to a probable link between dehydration sensitivity, seasonality, and timing of breeding bouts. Likewise, Preest and Pough (1989) conducted dehydration-performance experiments on *Anaxyrus americanus* that resulted in reliable and systematic reductions in locomotor performance. They found that distance traveled over 10-minute trial periods fell by 10-20 meters for every 10% reduction in hydration state across each of four experimental ambient temperature regimes. Watling and Braga (2015) investigated the role of dehydration sensitivity in driving dispersal limitations and habitat occupancy in 10 species of frogs across the families Hylidae, Microhylidae, Bufonidae, Leptodactylidae, and Strabomantidae, demonstrating that evaporative water loss impacted both geographic and within-habitat spatial distributions. Similarly, water loss vital limits and degrees of mass transfer were used as a correspondence metric in a study of frogs in the families Hylidae, Ranidae, and Scaphiopodidae that found strong correlations between habitat preference and desiccation sensitivity (Thorson and Svihla 1943).

When responding to the physiological challenges owed to dehydration risk, frogs are often forced to mediate their hydric state by way of adaptation. In terms of raw mass transfer, species across a range of families have been reported to tolerate up to 50% loss of their original body mass in a state of desiccation whereas others tolerate substantially lower vital limits (Thorson 1955; Titon et al. 2010; Wygoda 1984). Many of these adaptations are in response to abiotic habitat characteristics and are mediated by active behavioral hydro-regulation, and microhabitat utilization across their geographic range (Belmaker and Jetz 2011; Child 2008; Dodd 2010; Duellman 1999). For example, arboreal frogs in the families Hylidae and Rhacophoridae have been shown to increase their resistance to water loss using specialized skin secretions that prevent them from losing free water to the environment (Lillywhite 2006). Desert frogs in the families Hylidae, Myobatrachidae, Pyxicephalidae, and Schaphiopodidae conglomerate layers of shed skin and mucus to form a protective cocoon that prevents them from losing water to the substrate with a resistance rate 50 times greater than their normal (Lillywhite 2006; Loveridge and Crave 1979; Loveridge and Withers 1981; Withers 1995). In contrast to strategies of direct desiccation mitigation, many desert frogs demonstrate water balance

adaptations wherein they exhibit rapid rehydration capability compared to frogs in more temperate environments (Bentley et al. 1958; Christensen 1974; Titon et al. 2010; van Berkun et al. 1982; Warburg 1971).

Considering both the aforementioned plasticity in desiccation tolerance across anuran lineages and the plethora of strategies used to mitigate dehydration, lineage-specific investigations into amphibian water loss and associated ecological dynamics are of utmost importance. In recent years, investigators have increasingly endeavored to employ complex mechanistic biophysical modeling (e.g., Kearney and Porter 2009) as a tool for spatially profiling species distributions as they apply to disease risk (Sonn et al. 2020), habitat suitability (Bartelt et al. 2010), invasion and migration potential (Kearney et al. 2008), and differential niche use (Riddell and Sears 2015) within an integrative eco-physiological framework. However, despite the clear importance of water balance in the lives of anurans, many ecological and environmental niche modeling efforts on amphibians primarily focused on profiling and partitioning the *thermal* niche (e.g., Blank and Blaustein 2012; Cunningham et al. 2016; Groff et al. 2014; Searcy and Shaffer 2014) without directly measuring *in situ* water loss resistances and differential mass transfer, nor incorporating specific metrics of water loss rates into their models. Several studies have indeed underscored the critical importance of aiming to integrate more precise water loss estimates when constructing mechanistic niche- and other ecologically predictive models for amphibian taxa exhibiting variable habitat use and/or poorly understood ecological interactions (Bartelt et al. 2010; Kearney et al. 2008; Lertzman-Lepofsky et al. 2020; Riddell and Sears 2017). Moreover, taxa distributed throughout the tropics have described as particularly vulnerable to climate warming (Huey et al. 2009; Huey et al. 2012).

In the present study, we aim to investigate the eco-physiological characteristics of Sulawesi fanged frogs (genus: *Limnonectes*, family: Dicroglossidae). Frogs of the genus *Limnonectes* are widely distributed across mainland and insular Southeast Asia and currently comprise 77 species. Next to nothing is known about the ecological dynamics that drive their diversity, behavior, and varied life histories. To compound this dearth of baseline ecological information, much taxonomic uncertainty exists within the clade; however, new species are now being delimited and described at a rapid pace (Aowphol et al. 2015; Frederick et al. 2022a; Frederick et al. 2022b; Iskandar et al. 2014; Köhler et al. 2021; McLeod 2008; McLeod 2010; McLeod 2011; McLeod 2015; Phimmachak et al. 2018; Stuart et al. 2006; Stuart et al. 2020; Ye et al. 2007; Yodthong et al. 2021). Many fanged frogs are lowland or montane stream obligates, although leaf litter specialists are also common, and the assemblage is known to exhibit a diversity of derived reproductive modes (Emerson and Inger 1992; Gillespie et al. 2004; Goyes Vallejos et al. 2017; Goyes Vallejos et al. 2018; Iskandar et al. 2014). Setiadi et al. (2011) argued that the *Limnonectes* assemblage on the Indonesian island of Sulawesi constitutes an adaptive radiation, an assessment that was based primarily on the ~1000-fold variation in adult body mass exhibited by these frogs, as well as the propensity for multiple species to occur in sympatry. Only five described *Limnonectes* species occur on Sulawesi, but our own preliminary research suggests that the Sulawesi *Limnonectes* assemblage is much richer than even the 13 species that Setiadi et al. suggested and is likely composed of more than 40 species that iteratively partition niches in cohorts of 4–10 sympatric species. In general, niche partitioning occurs as follows: (1) giant-bodied frogs measuring up to 300 mm in snout-vent length (SVL) and weighing between 200g and 2–3kg occupy a mostly aquatic-, apex predator niche space within broad, fast-moving lowland and montane streams where they are particularly associated with rapids and waterfall

spray zones, (2) a number of mid-sized species between ~20-50mm and weighing 20g-80g occupy stream banks, overhanging logs, and mossy boulder outcroppings bordering headwater streams, and (3) cohorts of leaf litter specialists weighing 2g–15g occupy the microhabitat from 10–50 meters from offshoot channels, trickles, or seeps. As part of a long-term research program focusing on identifying the key axes of differentiation that characterize the Sulawesi *Limnonectes* putative adaptive radiation, we aimed to pursue biophysical mechanistic modeling as method for potentially detecting a signal of eco-physiology mediated lineage divergence across populations of fanged frogs. To facilitate this future research goal, we first aimed to collect proxy metrics of dehydration potential across the species assemblage in effort to test for any indication of differential water loss. We hypothesized that species more intimately tied to stream habitats would be more prone to dehydration when challenged with dry conditions, while species that spend substantial time in leaf-litter habitats away from streams would be more resistant to dehydration. Thus, the large, in-stream "waterfall giants" would produce steeper water loss rates or under our experimental design when compared with the intermediate stream-size species, as well as the tiny leaf-litter specialists routinely found far from flowing water. We also predicted that non-sympatric frogs of similar niche occupancy and microhabitat preference would demonstrate more similar water loss than spatially co-distributed sympatric frogs because the fanged frog assemblage is comprised of regional cohorts of (different) fanged frog species that appear to distribute themselves across the same range of habitat types. We aimed to test these hypotheses by conducting water loss trials using a novel field-executed airflow dehydration *in situ*.

METHODS

Field Work and Sampling.—As part of an ongoing research program focused on the ecology and systematics of the Sulawesi *Limnonectes* assemblage, we mounted expeditions to four mountain complexes between 2016 and 2018. Our collecting sites included: Gunung Bawakaraeng (2,845 meters (m)) at the southern tip of the Southwest Peninsula, Gunung Latimojong (2,871 m) – part of the Rantemario mountain complex north of the Tempe Depression in Luwu Regency, Gunung Torompupu (2,470 m) in the Central Core near Lore Lindu National Park, and Gunung Dako (2,304 m) – situated on the Northern Peninsula of Sulawesi (Figure 1). These sites latitudinally span the island over roughly 700 kilometers (km) and provided ample opportunities to sample a wide array of endemic fanged frog species across independent elevational gradients. At each mountain locality, we opportunistically hand-captured frogs in- and around stream sites ranging between 200 and 2,000 m in elevation. Though we tentatively identified animals to species-level in the field, cryptic speciation is well-known across the Sulawesi fanged frog assemblage; thus, species assignments for each of our eco-physiological trial subjects were subsequently confirmed through multi-species coalescent phylogenomic methods (see Frederick et al. 2022). Our collection efforts resulted in trial cohorts collectively comprised of $n = 64$ frogs, and respectively demarcated into eight (four described and four undescribed) species complexes: *L. microtympanum* ($n = 7$; in-stream giant; G. Bawakaraeng), *L. arathooni* ($n = 10$; stream bank - leaf litter intermediate; G. Bawakareng), *L. 'sp. 2'*($n = 7$; stream bank - leaf litter intermediate; Latimojong), *L. 'sp. Inflatus'* ($n = 4$, in-stream giant; G. Latimojong), *L. 'sp. T Red'* ($n = 5$; leaf litter obligate; G. Latimojong), *L. 'sp. T Yellow'* ($n = 11$; leaf litter obligate; G. Torompupu and G. Dako), *L. larvaepartus* ($n = 10$; leaf litter obligate; G. Dako), and *L. heinrichi* ($n = 8$;leaf litter obligate; G. Torompupu and G. Dako)

(Figure 2). All expedition field work and logistics were conducted under permit from the Indonesian Ministry of Research, Technology and Higher Education (RISTEK) in collaboration with the Museum Zoologicum Bogoriense (MZB) and the Indonesian Institute of Sciences (LIPI). Prior to our field expeditions and eco-physiological trials, animal handling and experimental protocols were approved by the UC Berkeley Institutional Animal Care and Use Committee (Protocol: R279).

Water Loss Trials.—Animals were field tested for desiccation tolerance using a three-lane (independent) flow apparatus (Figure 3). Stage one of the apparatus consisted of three separate air pumps (Boyu Co.) connected to flow meters (Dakota Instruments) – ensuring equal air flow (6.4L/min at 0.012 MPa) through each lane. Stage two consisted of three eight-inch desiccation tubes each filled with 30 grams of Drierite (anhydrous calcium sulfate) color indicated desiccant. Stage three consisted of sealed 0.95 liter (L) polypropylene chambers. The experimental chambers in stage three housed either the live animal, an agar frog mold of similar size and mass to the study subject, or remained empty. The empty chamber acted as a secondary control to compare rate curves obtained from agar and live animal chambers against ambient conditions within the remaining final stage chamber. The agar model chamber acted as control to for comparing water loss rate curves of the animal against those obtained from a similar shaped object, composed of a material of known density and static desiccation rate. Though we aim to expand on the use of our agar model data in the future, the purposes of the models in the study were merely to ensure proper function of the flow apparatus by visually checking the raw data sensor data for apparatus function when the loggers downloaded. Nevertheless, we tried to mitigate potential sources of error between agar model surface properties and amphibian skin properties, and to represent the overall shape and body size differences across fanged frogs, we first created latex rubber molds (Mold Builder – Environmental Technologies Inc.) of *Limnonectes* museum specimens posed in water conservation posture to encompass the various species, size classes, and variations in skin rugosity. When casting the molds in the field, we boiled a solution of 147 grams of agar molecular genetics powder (Fisher Scientific) in one liter of water, poured the solution into the molds, and allowed the agar to set for 24 hours. After releasing the molds from their casts, we stored the molds in a water bath to promote maximum hydration of the medium prior to the start of our experimental trials. The final stage of the apparatus consisted of three additional polypropylene chambers that respectively housed one of three HOBO U23 Pro-V2 temperature (T) and RH sensors (Onset Computer Corp., model: U23-001A). Each stage of the respective flow lanes was connected with 4.76 mm or 6.35 mm lab-grade polyethylene airline tubing. The sensor chambers in the final stage included 2.5 mm venting hole at the anterior end of the chamber to maintain constant flow and avoid pressure build-up during a trial.

Prior to the start of water loss trials, animals were allowed to hydrate by housing them in large, inflated goldfish bag containing 1-4 fluid ounces of water for at least one hour. To mitigate undo stress any potential undue stress on the animals, each frog was only subjected to a single water loss trial. Desiccant tubes were refreshed with new Drierite for each trial and T/RH sensors were pre-programmed to record T/RH once per second. Trials were initiated by separately weighing both the animal and its corresponding agar mold on a digital scale, placing each in its experimental chamber, activating the T/RH sensors, recording the start time, and switching on power to the air pumps. Each trial was conducted for least 50 minutes (min). Upon cessation of each experimental trial, the stop time was recorded, both the animal and its corresponding agar

mold were removed from the experimental chambers, and subsequently weighed on a digital scale to determine the difference in water loss as indicated by the final mass value subtracted from the initial mass value. The frog would then be placed back into an inflated goldfish bag with 1-4 fluid ounces of water to rehydrate. Sensor data from each experimental chamber was then downloaded onto a laptop computer using a HOBO Waterproof Shuttle (Onset Computer Corp.) Animal chamber RH rate curves were visually inspected against control chamber rate curves using HOBOware Pro Software (Onset Computer Corp.) to confirm apparatus function and success of the trial. All resultant per-specimen data were collated in Excel (Microsoft) and subsequently processed using R Statistical Software (R Core Team, 2022).

Water Loss Rate Analyses.—Water loss is herein represented by two proxy metrics: (1) the change in frog chamber relative humidity over time at constant ambient temperature, and (2) the mass difference of a subject after exposure to a trial. The loggers recorded percent humidity each second, thus, for ease of data processing, we conducted our calculations based on averaged minute-long scales. Upon initial inspection of the water loss rate curves for all individuals, it became evident that after ~30-min of exposure to a desiccating environment, the frogs would assume water conservation posture in an effort to behaviorally mitigate water loss. This behavioral shift resulted in rate curves that terminated in long tails, thus representing exponential decay. In light on this, we fit each subject's curve to a standard exponential decay function in R using the 'drc' package and recorded the decay rate constant (k). We then compiled the k values for each trial subject across species and tested for significant differences between rates using a Kruskal-Wallis rank sum test.

Comparisons of Mass Loss.—To secondarily assess differences in water loss across species, we compared the difference between an animal's mass before and after the water loss trial. Because the trials occur over ~30 min, we expect that mass loss represents water loss, and we hereafter refer to this metric as mass transfer. We compiled the mass transfer data for trials across all species along with specimen metadata including collection site, snout vent length (SVL), pre-trial body mass, and percent of body mass lost during the trial. As the water loss statistical factors (e.g., hydrated body mass and post-trial body mass) were correlated and lacked statistical independence, we used principal components analysis to induce orthogonal transformations on the data. Subsequent to the model run, we extracted the variance components of each variable within the first two dimensions (cumulatively accounting for 94.64% of the total) to inform our interpretation of the model results. We then extracted the PC scores from the model and tested for significant differences in water loss potential between species in two ways. The first approach utilized all aforementioned input factors in a multivariate analysis of variance (MANOVA) on PC scores by species cohort, while the second focused on per-factor differences between species cohorts using individual Kruskall-Wallis tests.

RESULTS

Water Loss Rates.— Summary plots of the raw *Limnonectes* water loss trial data are given for each species depicted in Figures 4—11. Recall that the raw water loss curves for each trial / frog were also fitted to an exponential decay function. Table 1 lists the exponential decay rate values for all frogs within this study and Figure 12 depicts typical fitted exponential decay curves for each species (selected by the nearest-neighbor k value to each species' average). When we tested

for significant differences between the decay rates, the Kruskal-Wallis test was highly significant at any level of α ($X^2 = 36.414$; $P = 2.29E^{-6}$). Regarding decay rates, recall that higher absolute values indicate a faster decay and lower absolute values indicate shallower decay. *L. heinrichi* (a stream bank obligate) had an average decay rate factor of $\bar{X}_k = -0.154$ with SD = 0.053. *L. arathooni* (also a stream bank obligate) had an average decay rate factor of $\bar{X}_k = -0.124$ with SD = 0.069. The last stream bank obligate we collected, *L. Sp. '2'* had an average decay rate factor of $\bar{X}_k = -0.162$ with SD = 0.138. Of the three leaf litter obligates investigated in this study, *L. Sp. T Red* had an average decay rate factor of $\bar{X}_k = -0.$ -0.070 with SD = 0.013, *L. Sp. T Yellow* had an average decay rate factor of $\bar{X}_k = -0.$ -0.070 with SD = -0.127, and *L. larvaepartus* had an average decay rate factor of $\bar{X}_k = -0.$ -0.282 with SD = -0.074. Of the in-stream giants, *L. Sp. 'Inflatus'* had an average decay rate factor of $\bar{X}_k = -0.$ -0.026 with SD = -0.019, and *L. microtympanum* had an average decay rate factor of $\bar{X}_k = -0.$ -0.028 with SD = -0.022.

Degrees of Mass Loss.—Average water loss given by the proxy of differential mass before and after the trials ranged between 0.432—3.195 g of water pulled from the animals as a result of the desiccation trials. In order from greatest to least mass transfer, raw water loss was greatest in *L. Sp. 'Inflatus'* ($\bar{X}$ = 3.195g; SD = 1.091), followed by *L. microtympanum* ($\bar{X}$ = 1.423g; SD = 0.348), L. Sp. '2' ($\bar{X}$ = 0.990g; SD = 0.272), *L. heinrichi* ($\bar{X}$ = 0.876g; SD = 0.374), *L. Sp. 'T Red'* ($\bar{X}$ = 0.716g; SD = 0.104), *L. Sp. 'T Yellow'* ($\bar{X}$ = 0.612; SD = 0.211), *L. larvaepartus* ($\bar{X}$ = 0.492; SD = 0.222), and lastly *L. arathooni* ($\bar{X}$ = 0.432; SD = 0.211). In general, small frogs that routinely occupy terrestrial (versus aquatic) niches gave up a substantially greater amount of water as a percentage of overall body mass, than did giant in-stream eco-morphs. The multi-species suite of mass transfer results along with body size are summarized in Table 2.

Principal components analysis on the mass transfer data yielded three factors (body mass, SVL, and raw mass transfer) comprising at least 25% of the overall variance to dimension one, respectively. Percent mass transfer accounted for ~80% of the overall variance explained by dimension two. Roughly 95% of the overall variance in the model was encompassed by the first two dimensions (Figure 13). MANOVA results on PC scores factored by species were highly significant at any level of α for the first and second principal component (PC1: $P = 8.042E^{-10}$, PC2: $P = 1.186E^{-4}$) underscoring the differential desiccation tolerance responses of fanged frogs across size classes. Univariate comparisons of the raw mass transfer data with Kruskal-Wallis tests corroborated our multivariate findings, in that, results were highly significant for each factor at any level of α (SVL: $P = 8.71E^{-04}$; body mass: $P = 1.25E^{-06}$, mass loss: $3.43E^{-05}$; and mean percent loss: $3.248E^{-06}$). Summary results of our mass transfer data are enumerated in Table 2.

DISCUSSION

The primary objective in this study was to collect and compare proxy metrics for evaporative water loss rates across eight Sulawesi fanged frog species and test for basic species-level differences in water loss using two approaches (rates and raw mass). Overall, we found significant differences in both water loss rate metrics between frogs occupying in-stream (*L. Sp. inflatus* and *L. microtympanum*), stream bank (*L. arathooni*, *L. heinrichi*, *L. Sp. '2'*), and leaf litter frogs (*L. Sp. 'T Yellow'*, *L. Sp. 'T Red'*, and *L. larvaepartus*). We predicted that the relationship between water loss rates would play out by way of species exhibiting loss on a

spectrum, whereby terrestrial frogs would demonstrate the least water loss and in-stream obligates would demonstrate the most – losing a substantial amount of their body water during the experimental trials. The primary results of the experiments, however, suggest the opposite pattern. In response to a dehydrating environment, the large in-stream frogs exhibited what appears to be water *conservation*, whereas more terrestrially obligate species freely gave up substantial amounts of water. For example, observe relatively low % body mass loss rates (Table 1) for *L. Sp. 'Inflatus'* and *L. microtympanum* – compared to those of leaf litter specialists like *L. Sp. 'T Yellow'* or *L. Sp. T Red*. Anecdotally, we did routinely observe the more aquatic frogs curling up into a classical anuran water conservation posture, whereas small terrestrial frogs were observed to remain active over the progression of the trial. That said, we explicitly did not conduct systematic focal sampling on the animals, thus we can only speculate that more aquatic-obligate animals were reacting conservatively in response to dehydration risk (and conversely, that leaf litter frogs were less stressed by the trial conditions). This pattern was maintained down the cascade of niche obligates. Stream bank frogs that can be found at a river or stream's edge (but avoid being directly in the center of a stream possibly due to predation pressure from the in-stream giants) demonstrated a medium level of dehydration compared to in-stream or leaf litter frogs. These results lend valuable insight into the degree to which fanged frogs *may* engage in behavioral hydro-regulation, an aspect of their life history that is as heretofore completely unknown. Given these observations, we suggest that future investigators consider the degree to which behavior might play into hydro-regulation. Substantive focal animal sampling that systematically tracks the both the instance of a physical posture shift and the comparative duration of this behavioral state during dehydration bouts would contribute much to our understanding of the interplay between regulatory behavior and environmental stress. In terms of differential mass water loss comparisons, our results clearly show that in-stream giant morphs only gave up between 1.7% and 2.5% of their body mass in water over the course of the trials, whereas the stream bank and leaf litter frogs gave up between 5% and 11% of their body mass in water. It is entirely possible that this could also be the result of surface area to volume ratio as it applies to water loss resistance – a cross-species comparison that we hope to incorporate in our downstream mechanistic and spatially explicit biophysical modeling efforts.

The findings herein are highly informative regarding our aim to better understand how variable life history strategies and niche occupancy may involve an interplay with the eco-physiological maintenance requirements of hydric state in an equatorial amphibian assemblage. Past research has certainly underscored that an amphibian's reliance on water availability for breeding and dehydration avoidance is constraint on their distribution throughout montane habitats and elevational gradients (Navas 2002). Moreover, co-varying factors like body size and mass are known to affect amphibian water balance, in that smaller animals are understood to lose water faster (or to a higher degree) than large ones (Peterman and Semlitsch 2014). Our decay rate and dehydration mass results are consistent with these prior findings. It is important to note that water balance in amphibians does not exist in a vacuum, and activity states / times, energy expenditure for reproductive requirements, metabolic rates, and temperature variability all play integral roles in the eco-physiological profile of amphibian life history (Peterman and Semlitsch 2014). Further, baseline dehydration tolerances definitively vary across species and populations of frogs, and the adaptive potential for any frog assemblage's water balance requirements could play out either through selection over evolutionary temporal scales or manifest *in situ* via plasticity (Vimercati et al. 2018). The mismatch between our original prediction that in-stream giant fanged frog morphs would be most susceptible to water loss while leaf litter frogs would

exhibit comparatively minute water loss, is certainly surprising, though we suspect that when tested using other physiological methods like performance or preference trials, this supposition could still be in line with the logic behind our initial hypothesis. Given the specific conditions of our experimental design, it makes intuitive sense that an aquatic animal would demonstrate compensatory activity states or behaviors when exposed to unfavorable conditions. Such compensatory acts would, in theory, not be exercised if they did not have some beneficial effect on some factor of the animal's eco-physiology or natural history. Congruently, rather than being better insulated from desiccating conditions via adaptations of the integument, the leaf litter obligates may have evolved higher water loss tolerances instead. Again, future work is needed to disentangle these possibilities from the results we've reported. Overall, we do find reasonable support for our final hypothesis, insofar as non-sympatric niche obligates exhibited decay rates more similar to each other than to sympatric congeners occupying nearby microhabitats. For example, the highest values of *k* were among *L. Sp. T Red*, *L. Sp. T Yellow*, *L. heinrichi*, and *L. arathooni*, all of whom are leaf litter or stream bank obligates. This suite of species represents the entire 700km latitudinal span of our survey sites and are not co-distributed in a single community. Likewise, the lowest rate decay factors that we found were in the two aquatic stream giants, *L. Sp. 'Inflatus'* and *L. microtympanum* and both of these frogs likewise occupy the same microhabitats yet are non-sympatric.

To better inform how we assign habitat obligations as they apply to water balance, future research efforts could implement a new suite of trials across fanged frog species and attempt to directly test locomotor performance under varying levels of hydric stress. Executing newly parameterized suites of trials would bolster our capability to describe fanged frog eco-physiology more robustly by facilitating the production of hydric performance curves, metrics of hydric preference, and theoretical optimality models. In the latter case, such investigations could attempt to expand upon a marginal value theorem ("-like") framework (Charnov 1976) that aims to calculate the asymptote (optimality state) of a curve describing mass transfer across time exposed to desiccating conditions, wherein the X_2 axis could describe activity time *not* engaging in water conservation behavior (see hypothetical example: **FIGURE 14**). Doing so could enable the explicit derivation of a set of species-specific metrics that quantitatively define the trade-off between a frog's terrestrial locomotor activity and dehydration risk – while also indicating a precise temporal window wherein one could expect that a frog should abandon activity in favor of water conservation or imminent re-hydration according to the "optimum decision rule" (for hypothetical model diagram, see Figure 14) (Davies et al. 2012). At present, we have yet to meet our long-term goal of producing mechanistic models that accurately reflect the eco-physiological drivers that likely acted as a primary catalyst for lineage divergence in this radiation; however, our results on the derivation of species-level water loss metrics were compelling. Along with the ideas for subsequent data acquisition outlined above, future research by way of fine-scale spatial- and biophysical modeling efforts will undoubtedly contribute even more to our understanding of the eco-physiological dynamics and mechanisms that resulted in this fascinating, niche-partitioned amphibian radiation.

FIGURES AND TABLES

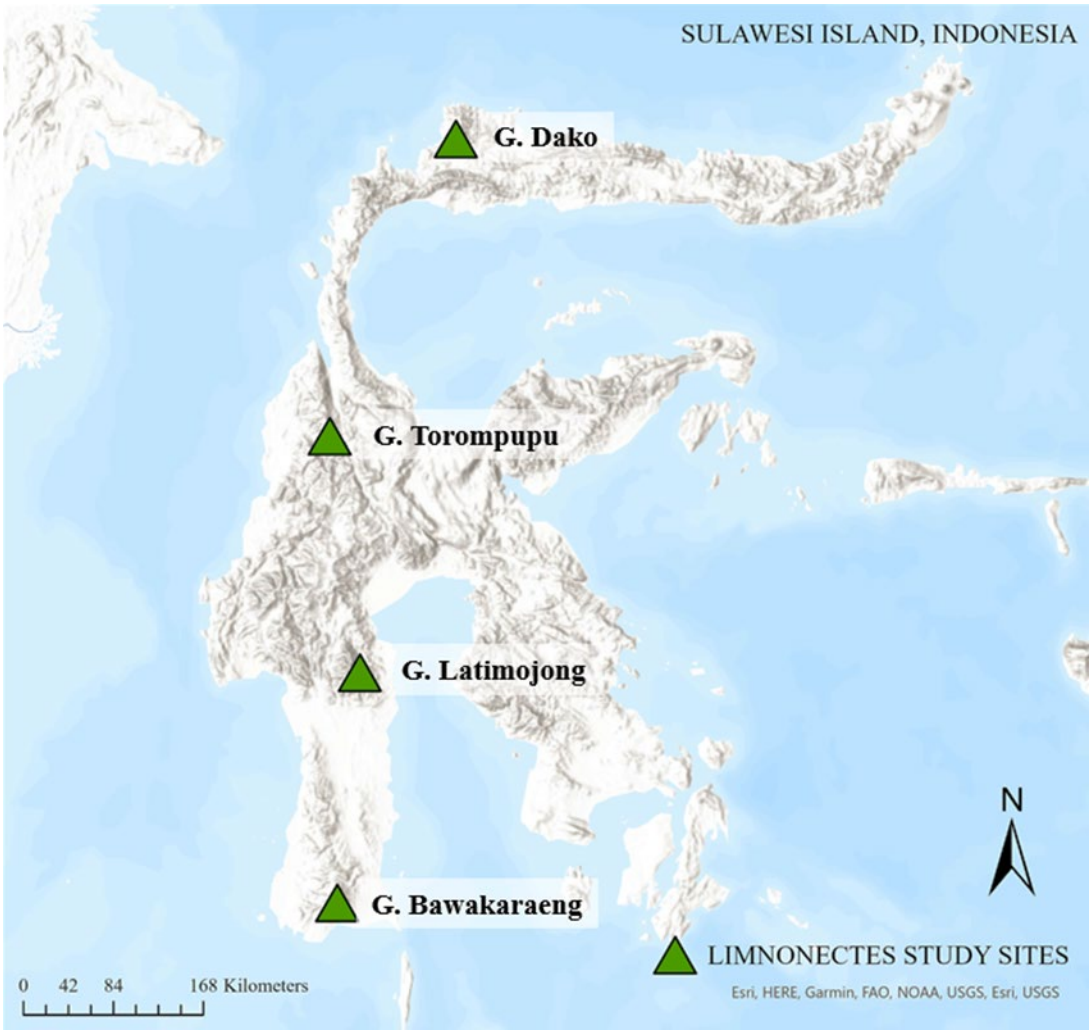

FIGURE 1—Survey and collection locations for our *Limnonectes* water loss investigations. *L. arathooni* and *L. microtympanum* were collected from G. Bawakaraeng. *L. 'Sp. T Red*, *L. 'Sp. 2'*, and *L. Sp. 'Inflatus'* were collected from G. Latimojong. *L. Sp. 'T Yellow'* and *L. heinrichi* were collected from G. Torompupu. *L. Sp. 'T Yellow'*, *L. Larvaepartus*, and *L. heinrichi* were collected from G. Dako.

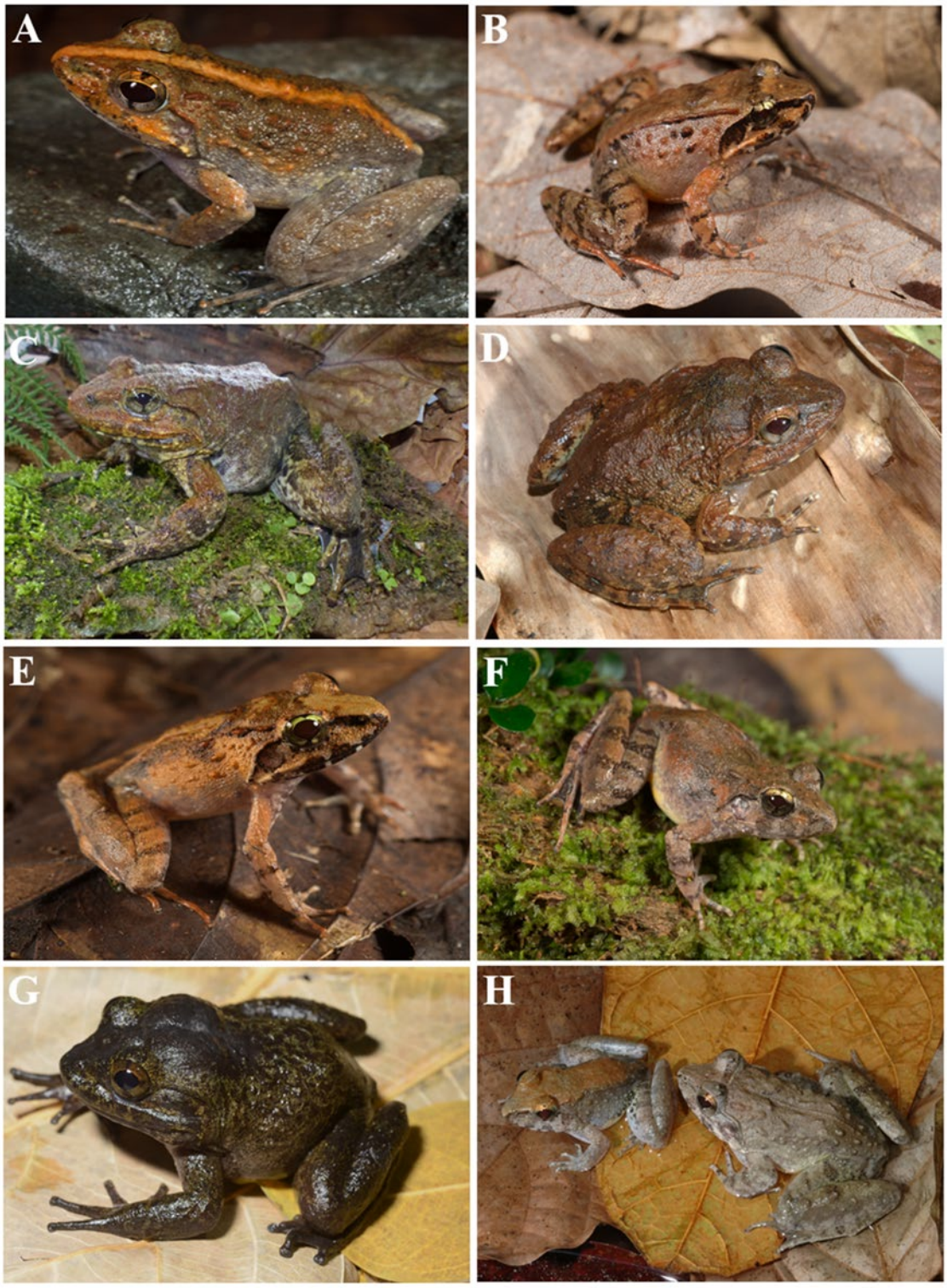

FIGURE 2 –The following eight species of fanged frogs were investigated in this study: (A) L. heinrichi, (B) *L. arathooni*, (C) *L. Sp. 'Inflatus'*, (D) *L. Sp. '2'*, (E) *L. Sp. 'T Red'*, (F) *L. Sp. 'T Yellow'*, (G) *L. microtympanum*, and (H) *L. larvaepartus.*

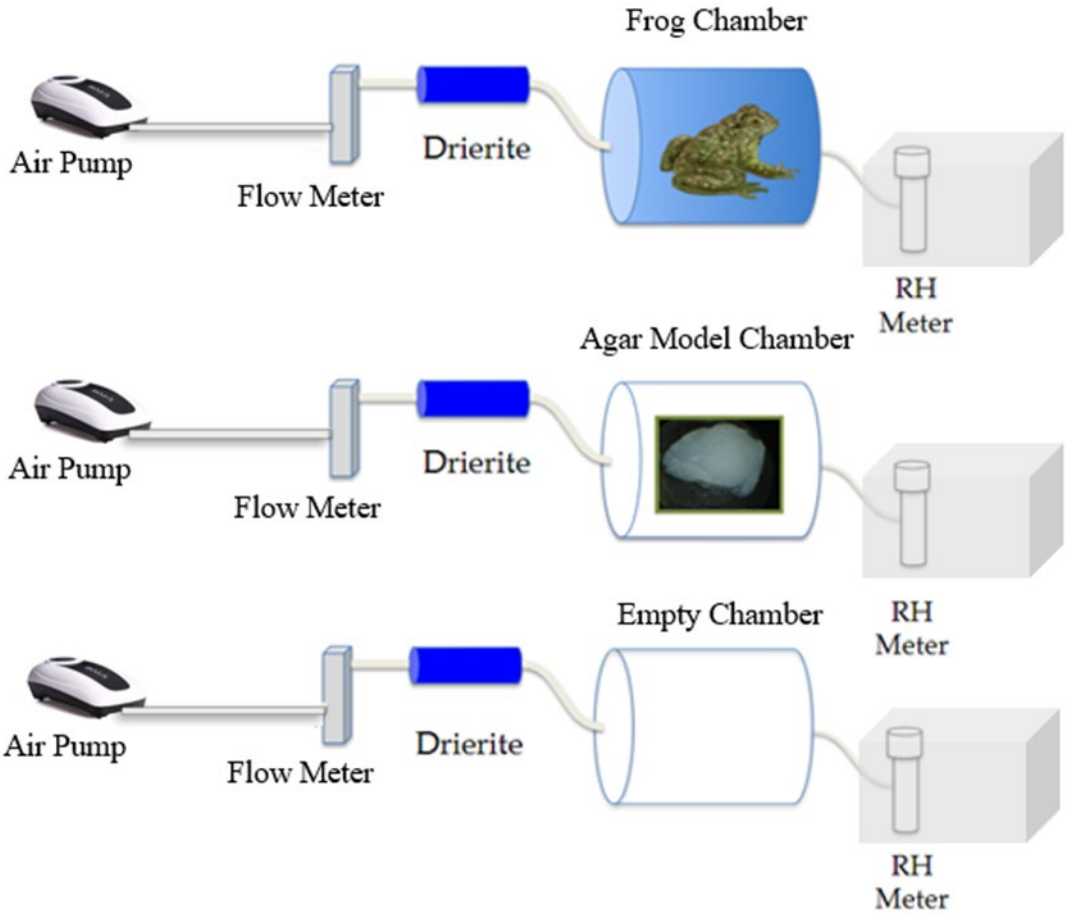

FIGURE 3 –Diagram of the flow-lane apparatus used in our experimental water loss trials. Air pumps were activated to force air (at equal flow rates) through desiccant tubes. In the first two flow lanes, dry air was subsequently pumped into respective chambers containing either a live frog or scale replica agar model of the trial species. The experimental chamber in the third lane remained empty as a control. In the final stage of each flow lane, temperature and relative humidity sensors measured the change in both factors at 1-sec intervals over the course of ~1-hr trials.

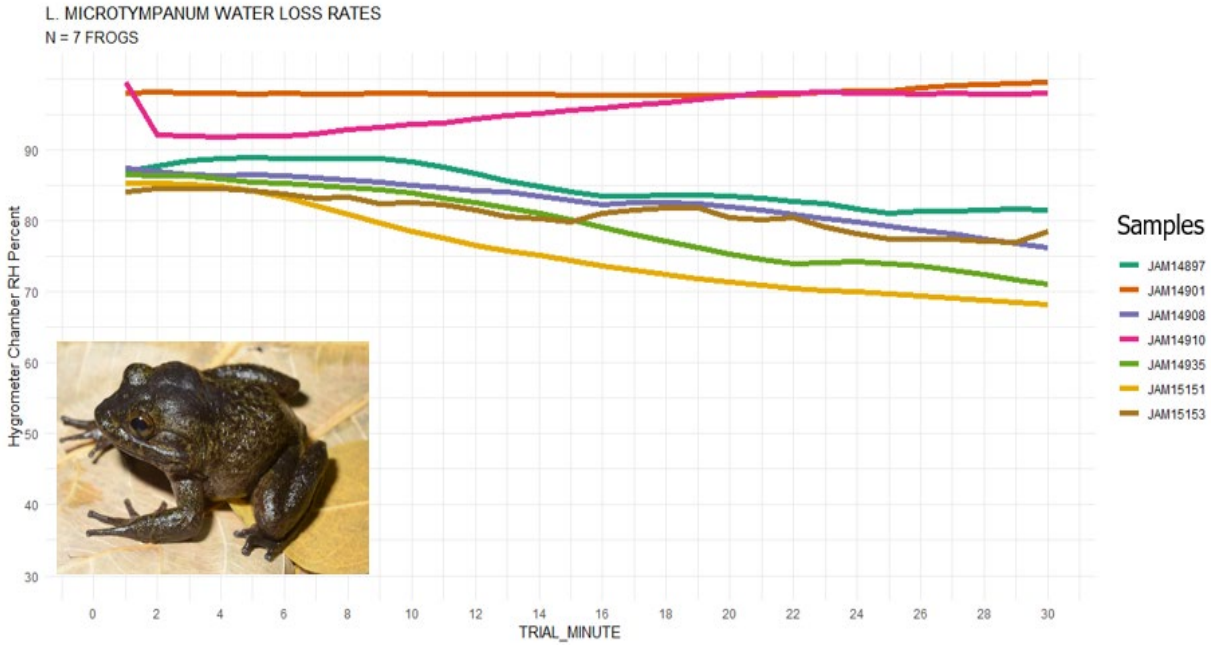

FIGURE 4 –Raw %RH data for *L. microtympanum* plotted in time series over the course of the first 30 minutes (to emphasize decay) of respective water loss trials. *L. microtympanum* is an in-stream giant fanged frog occupying broad fast-moving rivers, streams, and waterfalls.

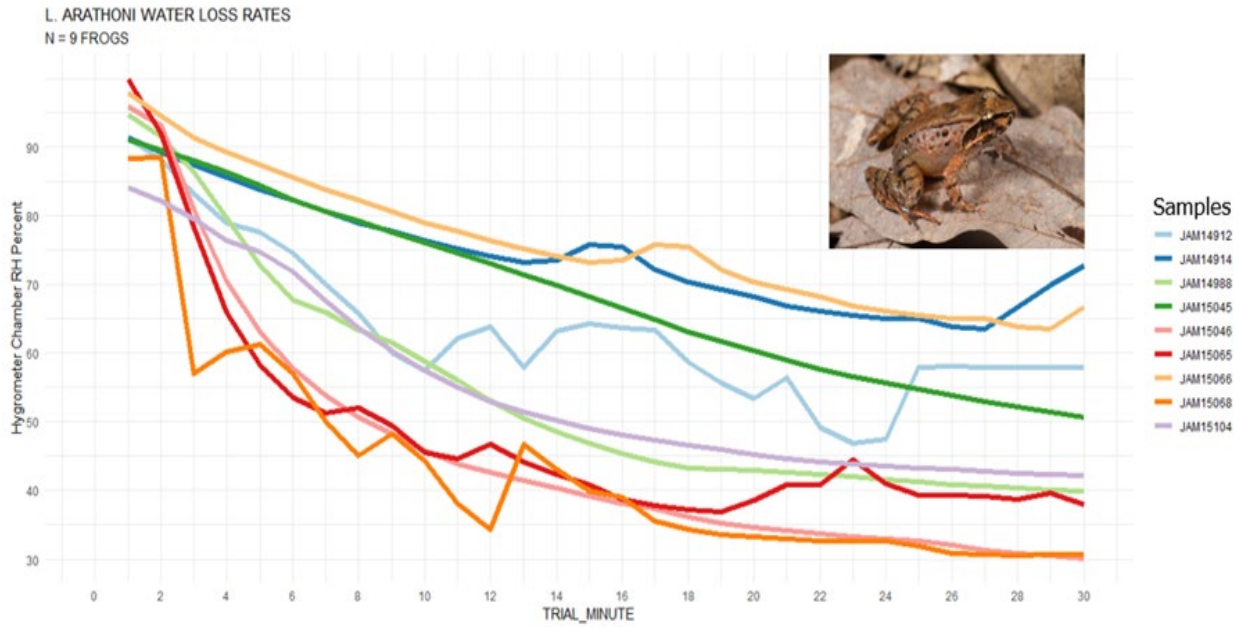

FIGURE 5 –Raw %RH data for *L. arathooni* plotted in time series over the course of the first 30 minutes (to emphasize decay) of respective water loss trials. *L. arathooni* is terrestrially breeding stream bank obligate.

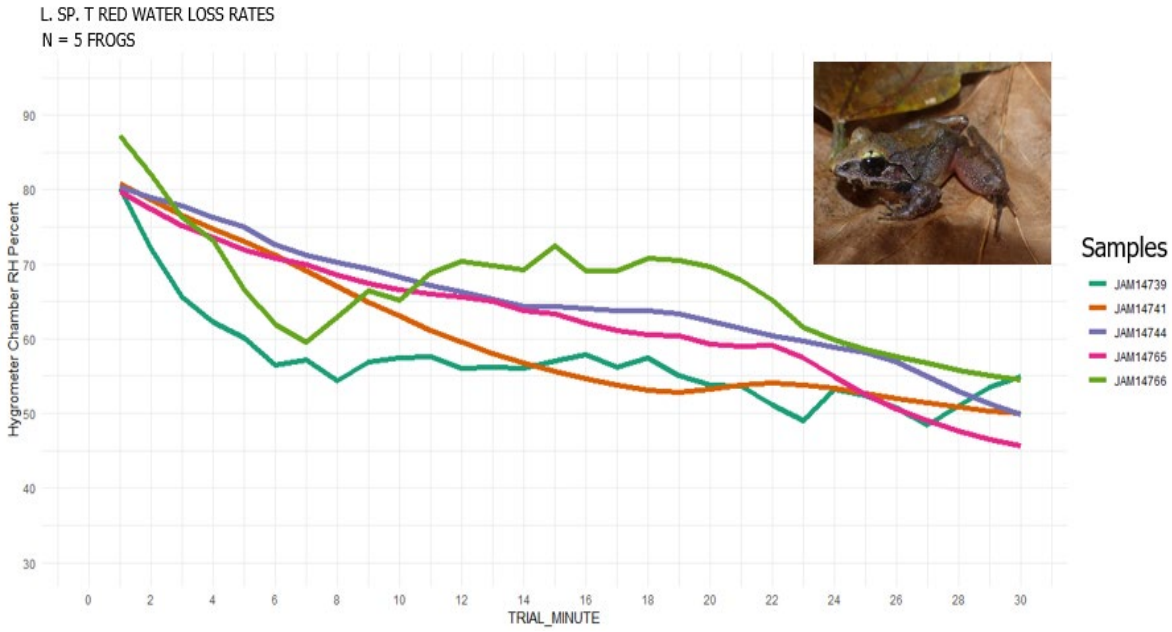

FIGURE 6 –Raw %RH data for *L. Sp. T Red* plotted in time series over the course of the first 30 minutes (to emphasize decay) of respective water loss trials. *L. Sp. T Red* is terrestrially breeding stream bank obligate.

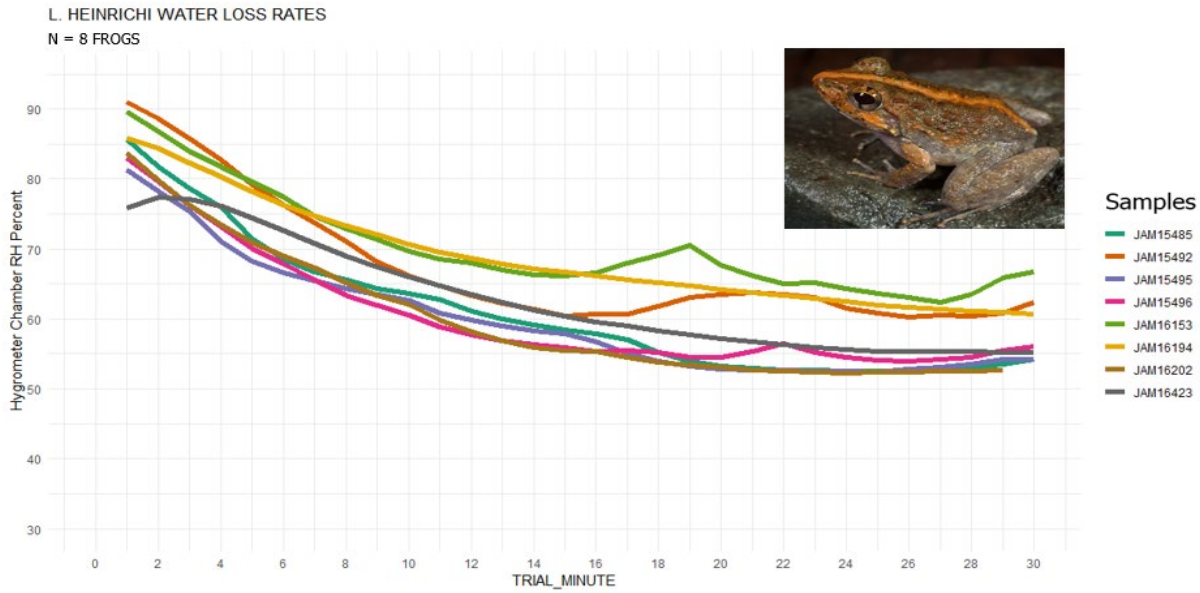

FIGURE 7 –Raw %RH data for *L. heinrichi* plotted in time series over the course of the first 30 minutes (to emphasize decay) of respective water loss trials. *L. heinrichi* is a mid-sized stream bank obligate.

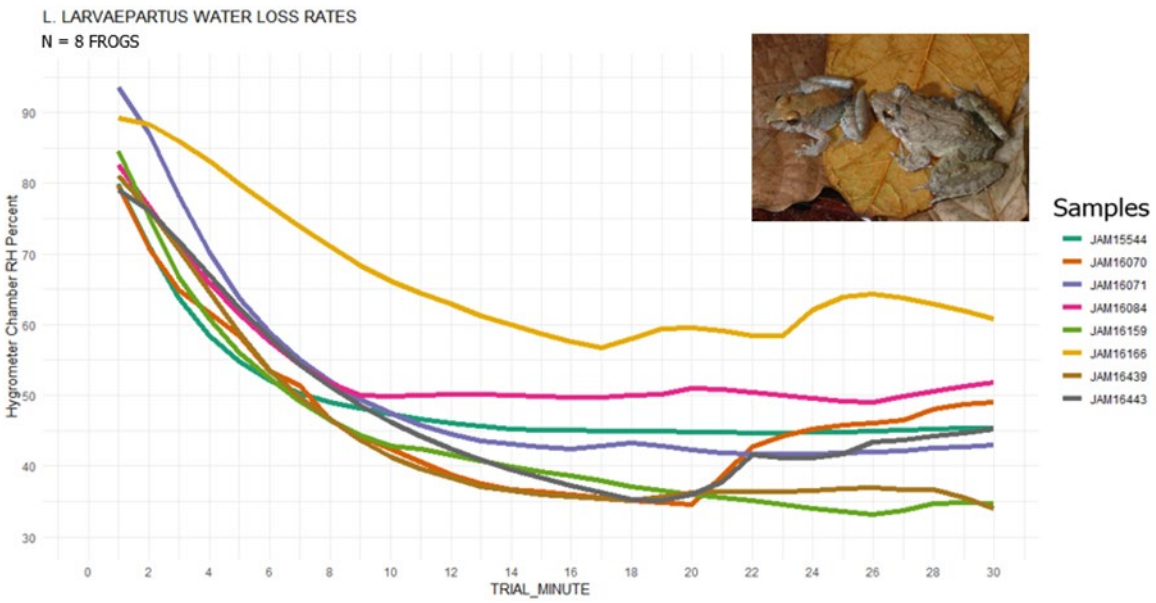

FIGURE 8 –Raw %RH data for *L. larvaepartus* plotted in time series over the course of the first 30 minutes (to emphasize decay) of respective water loss trials. *L. larvaeparetus* is a found in both leaf litter and stream bank habitats and has a unique tadpole live tadpole-bearing reproductive mode.

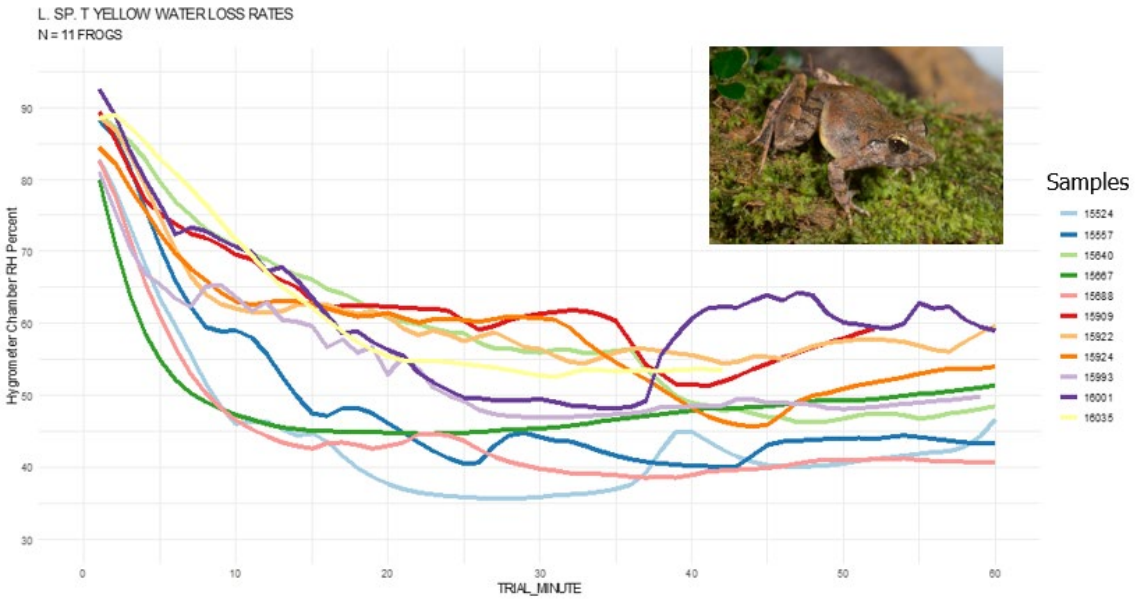

FIGURE 9 –Raw %RH data for *L. Sp. T Yellow* plotted in time series over the course of the first 30 minutes (to emphasize decay) of respective water loss trials. *L. Sp. T Yellow* is a strict leaf litter obligate, often found patrolling the forest floor.

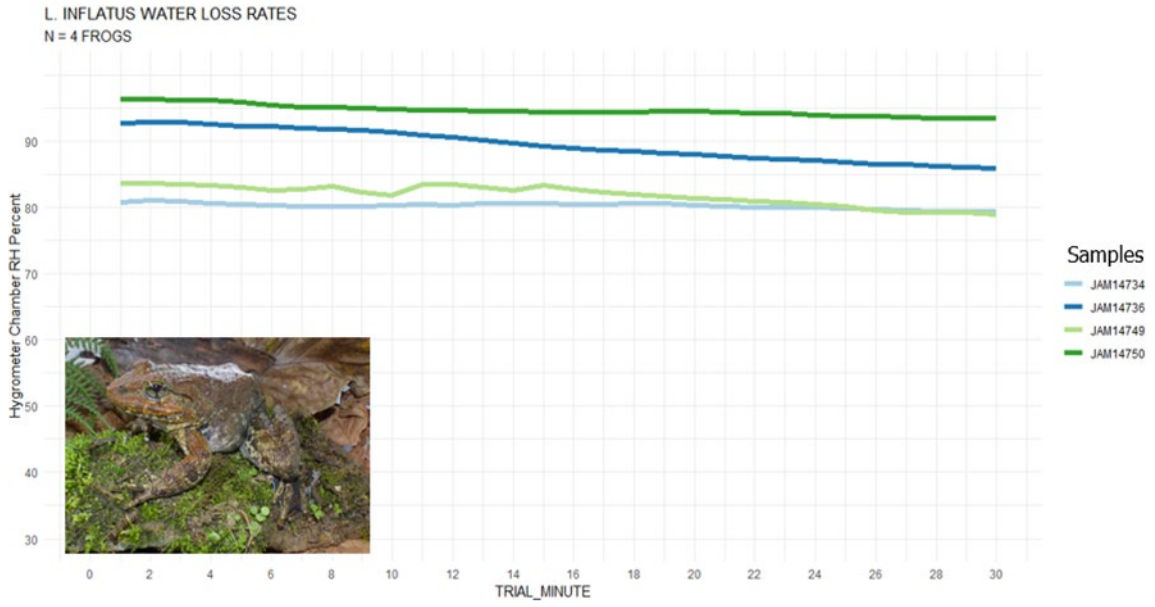

FIGURE 10–Raw %RH data for *L. Sp. 'Inflatus'* plotted in time series over the course of the first 30 minutes (to emphasize decay) of respective water loss trials. *L. Sp. 'Inflatus'* is a an in-stream is an in-stream giant fanged frog occupying broad fast-moving rivers, streams, and waterfalls.

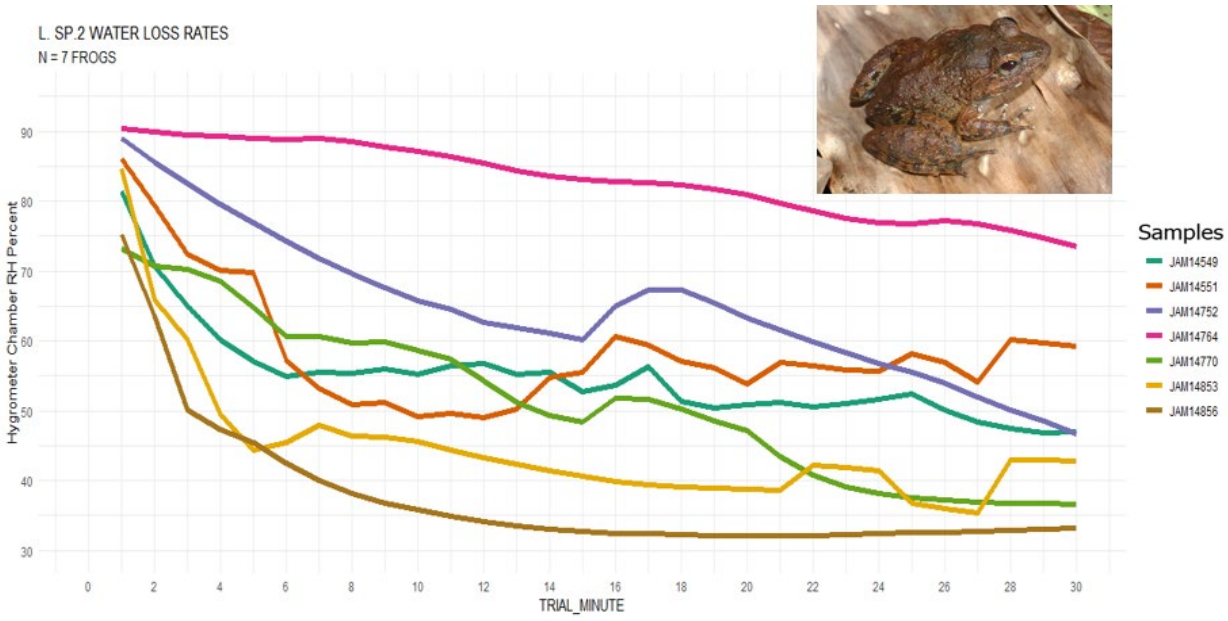

FIGURE 11–Raw %RH data for *L. Sp. '2'* plotted in time series over the course of the first 30 minutes (to emphasize decay) of respective water loss trials. *L. Sp. '2'* is a mid-sized stream bank obligate.

SPECIES	Specimen ID	*K* - Value
L. heinrichi	JAM16194	-0.098
L. heinrichi	JAM16202	-0.178
L. heinrichi	JAM16423	-0.056
L. heinrichi	JAM15492	-0.213
L. heinrichi	JAM15495	-0.162
L. heinrichi	JAM15496	-0.159
L. heinrichi	JAM16153	-0.154
L. heinrichi	JAM15485	-0.213
L. Sp. T Yellow	JAM15524	-0.189
L. Sp. T Yellow	JAM15640	-0.048
L. Sp. T Yellow	JAM15557	-0.136
L. Sp. T Yellow	JAM15667	-0.200
L. Sp. T Yellow	JAM15688	-0.200
L. Sp. T Yellow	JAM15919	-0.086
L. Sp. T Yellow	JAM15922	-0.164
L. Sp. T Yellow	JAM15924	-0.058
L. Sp. T Yellow	JAM15993	-0.084
L. Sp. T Yellow	JAM16001	-0.149
L. Sp. T Yellow	JAM16035	-0.091
L. larvaepartus	JAM15544	-0.383
L. larvaepartus	JAM16070	-0.370
L. larvaepartus	JAM16071	-0.284
L. larvaepartus	JAM16084	-0.228
L. larvaepartus	JAM16159	-0.201
L. larvaepartus	JAM16166	-0.199
L. larvaepartus	JAM16439	-0.250
L. larvaepartus	JAM16443	-0.340

TABLE 1–Exponential decay rate constants for water loss in eight species of *Limnonectes* fanged frogs. Each rate constant (*k*) was extracted from individual functions fit to the raw water loss rate data. Note: this table continues on the following page.

SPECIES	Specimen ID	*K* - Value
L. arathooni	JAM14912	-0.168
L. arathooni	JAM14941	-0.142
L. arathooni	JAM14988	-0.134
L. arathooni	JAM15045	-0.035
L. arathooni	JAM15046	-0.127
L. arathooni	JAM15065	-0.259
L. arathooni	JAM15066	-0.037
L. arathooni	JAM15068	-0.134
L. arathooni	JAM15104	-0.079
L. Sp. 'inflatus'	JAM14734	-0.042
L. Sp. 'inflatus'	JAM14735	-0.020
L. Sp. 'inflatus'	JAM14749	-0.002
L. Sp. 'inflatus'	JAM14750	-0.039
L. microtympanum	JAM14897	-0.024
L. microtympanum	JAM14901	-0.019
L. microtympanum	JAM14908	-0.010
L. microtympanum	JAM14910	-0.067
L. microtympanum	JAM14935	-0.036
L. microtympanum	JAM15151	-0.043
L. microtympanum	JAM15153	-0.001
L. Sp. T Red	JAM14739	-0.084
L. Sp. T Red	JAM14741	-0.076
L. Sp. T Red	JAM14744	-0.076
L. Sp. T Red	JAM14765	-0.048
L. Sp. T Red	JAM14766	-0.067
L. Sp. 2	JAM14549	-0.186
L. Sp. 2	JAM14551	-0.041
L. Sp. 2	JAM14853	-0.346
L. Sp. 2	JAM14856	-0.139
L. Sp. 2	JAM14752	-0.058
L. Sp. 2	JAM14764	-0.021
L. Sp. 2	JAM14770	-0.346

TABLE 1 (Continued)–Exponential decay rate constants for water loss in eight species of *Limnonectes* fanged frogs. Each rate constant (*k*) was extracted from individual functions fit to the raw water loss rate data.

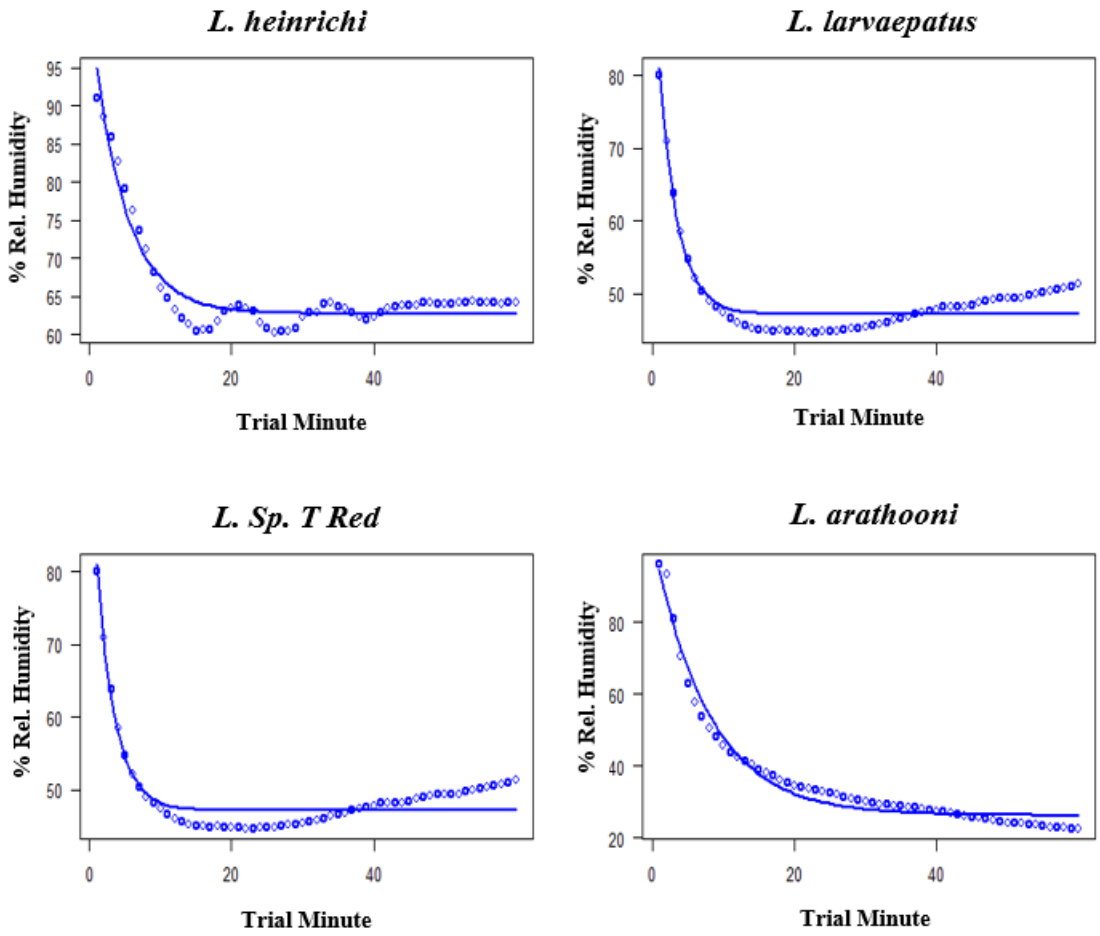

FIGURE 12– Typical fitted exponential decay curves for each species selected by the nearest-neighbor *k* value to each species' average. Dots reflect true (raw) data and solid lines reflect the fitted curve. The specific curves depicted represent exponential decay functions for JAM15496 (*L. heinrichi*), JAM 15544 (*L. larvaepartus*), JAM14744 (*L. Sp. T Red*), and JAM 15046 (*L. arathooni*). Note: this figure continues on the following page.

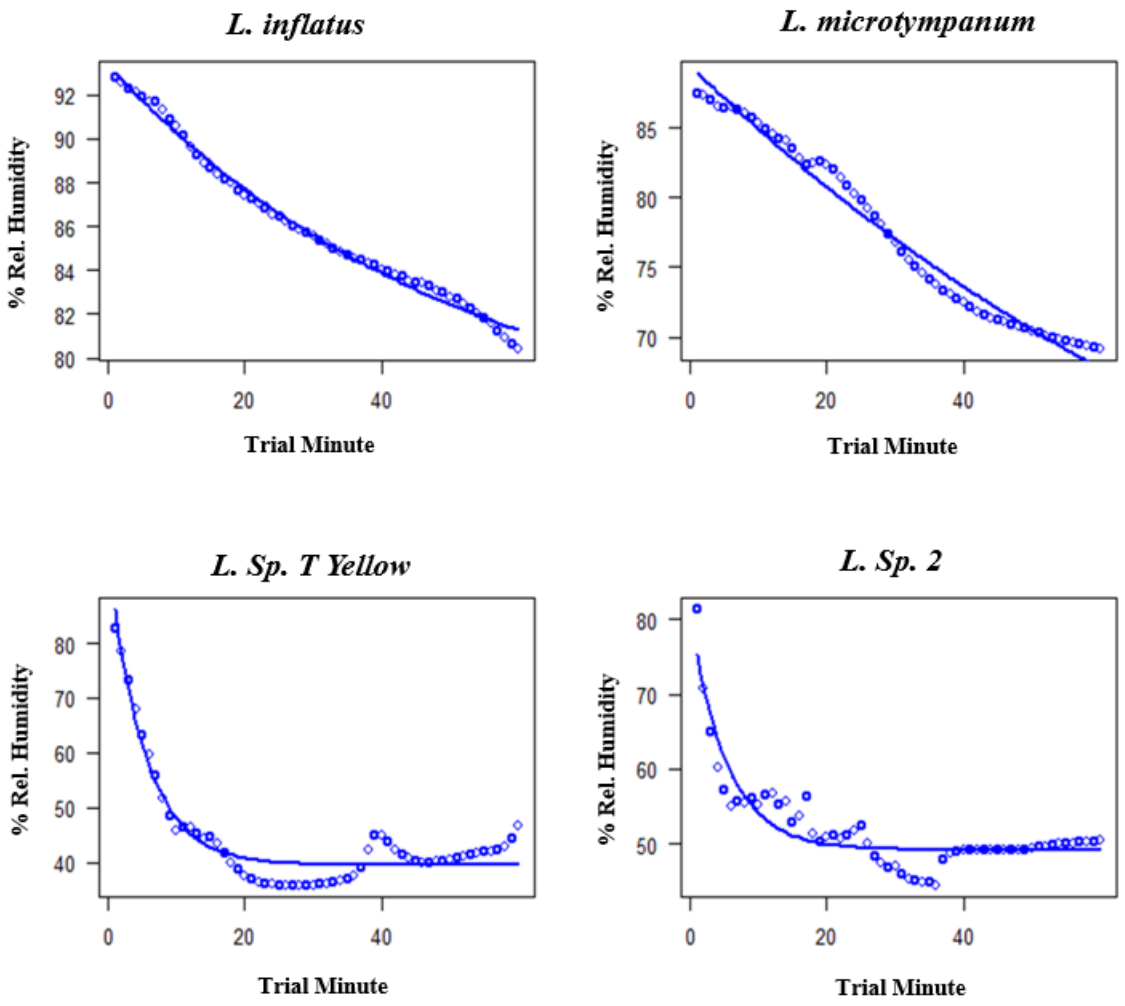

FIGURE 12 (Continued)– Typical fitted exponential decay curves for each species selected by the nearest-neighbor *k* value to each species' average. Dots reflect true (raw) data and solid lines reflect the fitted curve. The specific curves depicted represent exponential decay functions for JAM14735 (*L. Sp. 'Inflatus'*), JAM 14908 (*L. microtympanum*), JAM15524 (*Sp. T Yellow*), and JAM 14549 (*L. Sp. 2*).

Species [SVL (mm)]	Mass Transfer (g)	% Body Mass	Elevation Range (m)
***L. Sp. 'T Yellow'* [45.4]**	$\bar{X}$ = 0.612; SD = 0.211	7.836	770 — 1220
***L. larvaepartus* [47.1]**	$\bar{X}$ = 0.492; SD = 0.222	5.740	298 — 823
***L. microtympanum* [84.6]**	$\bar{X}$ = 1.423; SD = 0.348	2.483	1224 — 1738
***L. Sp. 'Inflatus'* [126.3]**	$\bar{X}$ = 3.195; SD = 1.091	1.733	1350 — 1354
***L. Sp. 'T Red'* [43.2]**	$\bar{X}$ = 0.716; SD = 0.104	11.050	1374 — 1381
***L. heinrichi* [59.5]**	$\bar{X}$ = 0.876; SD = 0.374	4.594	463 — 823
***L. Sp. '2'* [45.7]**	$\bar{X}$ = 0.990; SD = 0.272	11.557	538 — 1464
***L. arathooni* [36.6]**	$\bar{X}$ = 0.432; SD = 0.211	8.151	1539 — 1713

Species [Specimen ID]	Hydrated Mass (g)	Dehydrated Mass (g)	% Water Loss
L. heinrichi [JAM16194]	14.37	13.90	3.27
L. heinrichi [JAM16202]	20.74	20.33	1.98
L. heinrichi [JAM16423]	26.35	25.22	4.29
L. heinrichi [JAM15492]	27.45	26.09	4.95
L. heinrichi [JAM15495]	26.60	25.52	4.06
L. heinrichi [JAM15496]	28.86	27.88	3.40
L. heinrichi [JAM16153]	22.17	20.95	5.50
L. heinrichi [JAM15485]	3.87	3.51	9.30
L. Sp. T Yellow [JAM15524]	4.58	4.14	9.61
L. Sp. T Yellow [JAM15640]	14.55	13.53	7.01
L. Sp. T Yellow [JAM15557]	10.07	9.62	4.47
L. Sp. T Yellow [JAM15667]	9.18	8.44	8.06
L. Sp. T Yellow [JAM15688]	8.16	7.37	9.68
L. Sp. T Yellow [JAM15919]	5.74	5.23	8.89
L. Sp. T Yellow [JAM15922]	7.26	6.42	11.57
L. Sp. T Yellow [JAM15924]	7.47	6.76	9.50
L. Sp. T Yellow [JAM15993]	7.17	6.67	6.97
L. Sp. T Yellow [JAM16001]	6.61	6.16	6.81
L. Sp. T Yellow [JAM16035]	7.71	7.43	3.63

TABLE 2–Summary data (top panel), raw water loss data (middle panel) collected during dehydration experiments. Percent water loss values reflect the percent of the animal's body mass removed due to the dehydration trials. The final (bottom) panel reflects summary data on body mass and habitat-type per species. Note: this table continues onto the next two pages.

Species [Specimen ID]	Hydrated Mass (g)	Dehydrated Mass (g)	% Water Loss
L. larvaepartus [JAM15544]	6.85	6.27	8.47
L. larvaepartus [JAM16070]	3.22	2.88	10.56
L. larvaepartus [JAM16071]	6.83	6.48	5.12
L. larvaepartus [JAM16084]	5.18	4.89	5.60
L. larvaepartus [JAM16159]	9.96	9.39	5.72
L. larvaepartus [JAM16166]	14.47	14.13	2.35
L. larvaepartus [JAM16439]	11.35	10.64	6.26
L. larvaepartus [JAM16443]	6.95	6.79	2.30
L. larvaepartus [JAM16442]	12.16	11.65	4.19
L. larvaepartus [JAM16488]	10.85	10.01	7.74
L. larvaepartus [JAM16441]	10.71	9.90	7.56
L. arathooni [JAM14912]	3.56	3.27	8.15
L. arathooni [JAM14941]	2.97	2.75	7.41
L. arathooni [JAM14988]	3.32	2.80	15.66
L. arathooni [JAM15045]	3.26	3.01	7.67
L. arathooni [JAM15046]	3.12	2.88	7.69
L. arathooni [JAM15065]	13.71	12.99	5.25
L. arathooni [JAM15066]	7.98	7.40	7.27
L. arathooni [JAM15068]	9.28	8.51	8.30
L. arathooni [JAM15104]	7.06	6.50	7.93
L. Sp. 'inflatus' [JAM14734]	230.64	226.76	1.68
L. Sp. 'inflatus' [JAM14735]	104.48	103.03	1.39
L. Sp. 'inflatus' [JAM14749]	226.29	221.98	1.90
L. Sp. 'inflatus' [JAM14750]	160.06	156.92	1.96
L. microtympanum [JAM14897]	44.08	42.78	2.95
L. microtympanum [JAM14901]	41.09	39.63	3.55
L. microtympanum [JAM14908]	47.75	46.44	2.74
L. microtympanum [JAM14910]	37.20	35.72	3.98
L. microtympanum [JAM14935]	35.77	35.00	2.15
L. microtympanum [JAM15151]	193.54	191.53	1.04
L. microtympanum [JAM15153]	168.17	166.54	0.97
L. Sp. T Red [JAM14739]	7.18	6.49	9.61
L. Sp. T Red [JAM14741]	8.75	8.14	6.97
L. Sp. T Red [JAM14744]	5.08	4.28	15.75
L. Sp. T Red [JAM14765]	5.31	4.70	11.49
L. Sp. T Red [JAM14766]	7.61	6.74	11.43

TABLE 2 (Continued)– Summary data (top panel), raw water loss data (middle panel) collected during dehydration experiments. Percent water loss values reflect the percent of the animal's body mass removed due to the dehydration trials. The final (bottom) panel reflects summary data on body mass and habitat-type per species. Note: this table continues onto the next two pages.

Species [Specimen ID]	Hydrated Mass (g)	Dehydrated Mass (g)	% Water Loss
L. Sp. 2 [JAM14549]	18.00	16.80	6.67
L. Sp. 2 [JAM14551]	10.50	9.50	9.52
L. Sp. 2 [JAM14853]	7.59	6.12	19.37
L. Sp. 2 [JAM14856]	3.28	2.68	18.29
L. Sp. 2 [JAM14752]	10.50	9.52	9.33
L. Sp. 2 [JAM14764]	14.19	13.50	4.86
L. Sp. 2 [JAM14770]	7.70	6.71	12.86

Species [SVL (mm)]	Average Mass (g)	Habitat Tpe
***L. Sp. 'T Yellow'* [45.4]**	$\bar{X}$ = 8.045; SD = 2.625	Leaf litter obligate
***L. larvaepartus* [47.1]**	$\bar{X}$ = 9.168; SD = 3.845	Leaf litter obligate
***L. microtympanum* [84.6]**	$\bar{X}$ = 81.086; SD = 68.665	In-stream giant
***L. Sp. 'Inflatus'* [126.3]**	$\bar{X}$ = 180.368; SD = 60.021	In-stream giant
***L. Sp. 'T Red'* [43.2]**	$\bar{X}$ = 6.786; SD = 1.564	Leaf litter obligate
***L. heinrichi* [59.5]**	$\bar{X}$ = 21.301; SD = 8.464	Stream bank obligate
***L. Sp. '2'* [45.7]**	$\bar{X}$ = 10.251; SD = 4.793	Stream bank obligate
***L. arathooni* [36.6]**	$\bar{X}$ = 5.938; SD = 3.840	Stream bank obligate

TABLE 2 (Continued)– Summary data (top panel), raw water loss data (middle panel) collected during dehydration experiments. Percent water loss values reflect the percent of the animal's body mass removed due to the dehydration trials. The final (bottom) panel reflects summary data on body mass and habitat-type per species. Note: this table continues onto the next two pages.

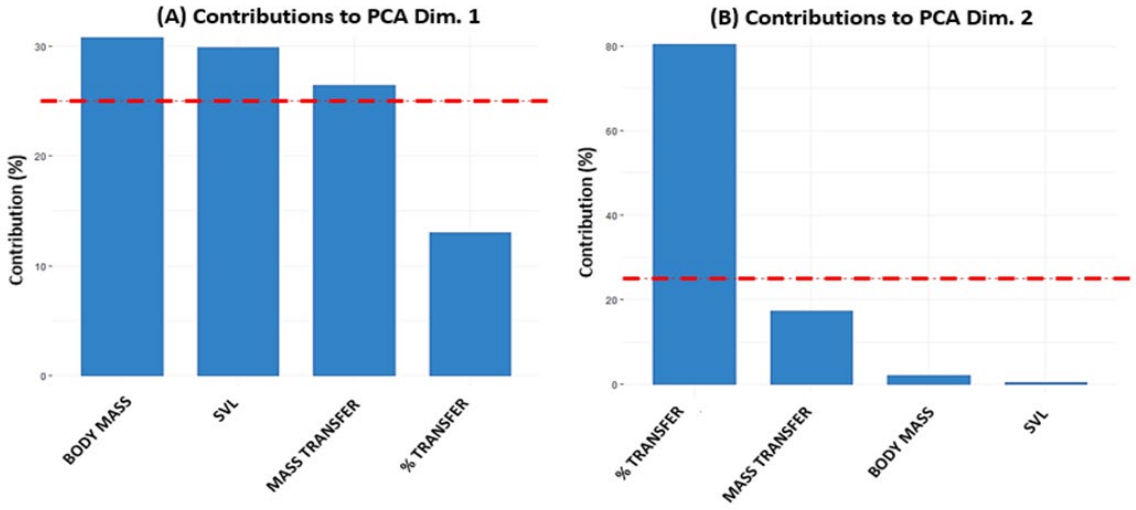

FIGURE 13–Histograms depicting variance contributions to the first two (mass) water loss PCA dimensions. Red dashed lines serve as a waypoint to identify which factors contributed to at least 25% of the overall variance in either the first or second orthogonal dimension.

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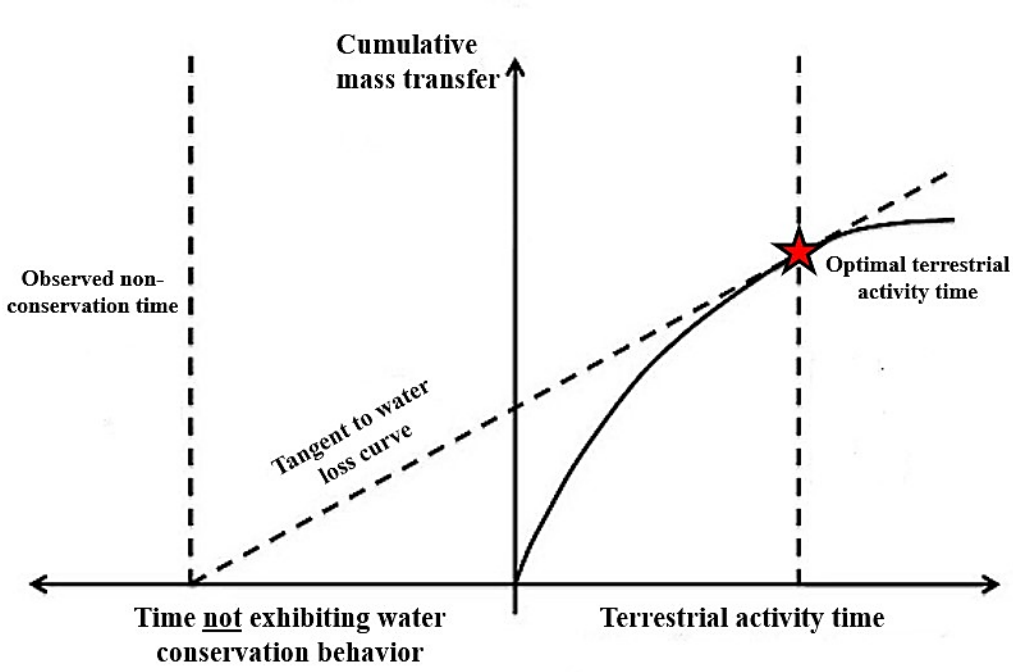

FIGURE 14–Diagram of a hypothetical marginal value theorem-based optimality model whereby future research efforts could attempt to quantify the trade-off among a frog's activity state, hydric mass loss, and need for proactive hydro-regulation.

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