TAXONOMY, SYSTEMATICS, BIOGEOGRAPHY, AND CONSERVATION OF TOADS (ANURA: BUFONIDAE) IN THE SUNDA SHELF OF INDONESIA

By

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DISSERTATION

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ABSTRACT

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Supervising Professor: Eric N. Smith

After Van Kampen's 1905–1911 visit to Indonesia culminating in his *Amphibians of the Indo-Australian Archipelago* (1923), there has been no extensive herpetological exploration and compendium for Indonesia, particularly for Sumatra, Java, and Kalimantan. This lack of herpetological surveys has resulted in recent molecular studies from Southeast Asia lacking samples from Sumatra, Java, and Kalimantan, and subsequent underestimation of herpetofaunal diversity, inaccuracies in phylogeny and biogeography, and lack of massive conservation status assessments. This is truer for the not so aesthetically appealing and rather warty bufonid toads of Indonesia, which have received very little attention from the small scientific community who study the herpetofauna in Sumatra, Java, and Kalimantan. Herein I address those shortcomings by generating comprehensive molecular datasets to study bufonid toads in the Sunda Shelf. I leveraged molecular, morphological, bioclimatic, geographic, and habitat data to discover genetic and distributional patterns of bufonid diversity. My study discovered one of the most elusive Southeast Asian bufonid genus, one endemic to Sumatra—*Sigalegalephrynus.* With molecular, morphological, bioclimatic, and acoustic data, I describe six *Sigalegalephrynus* species, five now provisionally placed to the IUCN Red List category of "Endangered". I also investigated the phylogeny and distribution of two other genera—*Phrynoidis* and *Leptophryne* and identified multiple candidate undescribed species

in both of these genera. This study will benefit future studies of bufonid toads in the Sundaland region and will serve as a framework for future molecular studies of Asian Bufonidae.

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DEDICATED TO MY FAMILY

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CHAPTER ONE

General Introduction

The Discovery of new species and biologically independent evolutionary units is important for the proper estimation of global flora and fauna and their conservation. Currently, global biodiversity is facing human-induced global sixth mass extinction (Barnosky *et al.* 2011, Ceballos *et al.* 2015, Ceballos *et al.* 2017). Global biodiversity hotspots are especially vulnerable to these anthropogenic threats and demand more conservation efforts from us than before. However, priority in conservation and sustainable use of biodiversity depends on the proper identification of species and/or populations and assessment of their threats of extinction. The identification of species requires proper integration and analysis of genetic, morphological, behavioral, distributional, and bioclimatic data (de Quirez 2017).

The Sundaland is one of the world's main biodiversity hotspots in Southeast Asia and encompasses the southeast extension of continental Asia and all the small and large islands—Sumatra, Java, and Borneo—west of Wallace's line. Unfortunately, like other global biodiversity hotspots, the Sundaland is not immune to anthropogenic threats of extinction. It is one of the most vulnerable ecosystems of the world and facing the highest deforestation rate (particularly, Sumatra; Margono *et al.* 2014). In the Sundaland, Sumatra and Java are two major islands where biodiversity exploration, particularly herpetofaunal exploration has been very limited since 1923 which has resulted in the underestimation of species diversity and lack of information on herpetofauna in those islands and (Harvey *et al.* 2017a 2017b, Sarker *et al.* 2019). For example, in the last 17 years, many herpetological expeditions have been conducted on Malaysia (peninsular and insular), resulting in the discovery of 78 new amphibian species, more than twice of the new species discovery in Java, Sumatra and

Indonesian Borneo (Kalimantan) combined (33 species; AmphibiaWeb 2020) although the size of Java, Sumatra and Indonesian Borneo combined is much larger than that of all of Malaysia. This discrepancy is directly ascribable to the lack of field expeditions in Sumatra, Java, and Kalimantan. For instance, from 2015 until now more amphibian species have been described from Indonesia (Java, Sumatra, and Kalimantan) than Malaysia (21 from Indonesia and 17 in Malaysia). And, most of this increase in new species discovery from Indonesia is a result, directly or indirectly, of the National Science Foundation international collaborative effort among The University of Texas at Arlington—UTA, Brawijaya University—UB, and the Indonesian Institute of Sciences—LIPI. An effort resulting in extensive herpetological surveys in Sumatra, Java, and Borneo, Indonesia from 2013 to 2016. In fact, out of 21 new Indonesian amphibian species, 13 species and one new genus have been described from the aforementioned research group. In terms of new toad species (Bufonidae) discovered from the region since 2017, this group discovered as many new species (6 species) from Java and Sumatra as have been discovered from Malaysia (AmphibiaWeb 2020).

I chose the amphibian family Bufonidae of the Sundaland for my dissertation research for several reasons:

1) Bufonidae is one of the speciose families in Southeast Asia—currently, representing ten genera and 74 species. Except for *Sigalegaelphrynus* and monotypic genus—*Sabahphrynus,* each genus is widely distributed among the insular and peninsular habitats. *Sigalegaelphrynus* is endemic to Sumatra and *Sabahphrynus* restricted to Sabah province of Malaysian Borneo (Frost 2020). This complex distribution offers largely a unique setting to study biogeography and distribution patterns of species.

2) Bufonidae is one of the least studied amphibian group in Sumatra and Java and diversity in the Bufonidae family is largely unexplored. For example, from 2004 to

2016 only two new species were described by two research groups, whereas from Malaysia 12 new species were described by eight research groups (Sarker *et al.* 2019, AmphibiaWeb 2020).

3) Currently, the deforestation rate in Indonesia (particularly in Sumatra) is highest in the world, and wildlife species are facing most probably the highest extinction rate (Margono *et al.* 2014). Thus, the assessment of herpetofaunal diversity is urgent for their conservation and proper management plan.

4) Most of the phylogenetic and phylogeographic studies of amphibian fauna of Southeast Asia failed to incorporate a wide range of molecular data from Sumatra, Java, and Indonesian Borneo (Kalimantan) which resulted in inaccurate phylogeny and evolutionary relationships among different taxa (Smart *et al.* 2017, Sarker *et al.* 2019).

This is the first study that used a comprehensive sampling of bufonid taxa across Sumatra and Java and some localities of Kalimantan. Coupled with molecular data produced for this study and data from the online data repository—GenBank—this study represents a robust dataset to date to study phylogeny and phylogeography of bufonid genera of Southeast Asia.

For my dissertation research, I used morphological, molecular (mitochondrial DNA), behavioral (mating call), distributional, and geospatial (environmental niche modeling) data to describe new species, identify current distribution through environmental niche modeling and performed conservation status assessment of propose IUCN Red List category for new species.

For my second chapter, I used one fragment of the 16S rRNA gene to explore molecular systematic relationships among several populations of *Sigalegalephrynus*—

recently discovered endemic genus from Sumatra. I combined acoustic, bioclimatic, and morphological evidence to describe three new species of this genus. I also used the IUCN recommended guidelines to assess IUCN Red List Status of all five species of *Sigalegalephrynus.* This chapter has been published in *Zootaxa* (Sarker *et al.* 2019).

In 2019 during revising pur collection of small toads, we came across another morphologically distinct *Sigalegalephrynus* specimen which represent a separate evolutionary distinct lineage. My third chapter is based on the finding of this new lineage. I employed two complete mitochondrial genes (mtDNA: *12S* rRNA and *16S* rRNA genes along with the flanking $tRNA^{val}$ gene), two nuclear genes (nuDNA: *NCX1* and *POMC*), and micro-CT scan dataset to elucidate more robust evolutionary relationships among six major lineages of *Sigalegalephrynus*. Other than a small fragment of *12S* (~450bp) and *16S* (~500bp) all of the sequences were generated for this project only and have not been used before. This chapter is prepared for *Zootaxa* and the coauthors are revising the manuscript. It will be submitted for publication very soon. We choose *Zootaxa* as this journal offers scientific publication without any publication fees.

I studied the giant river toad genus *Phrynoidis*—one of the widely distributed genera in Southeast Asia for my fourth chapter. I used mensural data, two complete mitochondrial genes (mtDNA: *12S* and *16S* along with the flanking $tRNA^{val}$), and four nuclear genes (nuDNA: *CXCR4*, *NCX1*, *POMC*, and *RAG1*) to assess systematic relationships of different populations from all over Southeast Asia. Although my fourth chapter has been finalized for submission and has been formatted for *Zootaxa* for the sake of consistency in my dissertation chapters, I wish to submit it to another journal after.

I used *16S* rRNA DNA dataset from 91 individuals for another widespread Indonesian tree toad genus—*Leptophyne* for my fifth chapter. Currently, this genus consists of three

species, two of which are microendemic to two different mountains in Java. As per the IUCN Red List category, one of them—*L. cruentata*—is listed as 'Critically Endangered' and the other—*L. javanica*—as 'Data Deficient' by IUCN. The third species—*L. borbonica* is one of the most widely distributed and found in southern Thailand, Peninsular Malaysia, Borneo, Sumatra, and Java. I studied molecular evolutionary relationships of these species and most of the populations of Sumatra and Java by employing phylogenetic and molecular divergence analysis. This chapter also will be submitted to a journal for publication after a major revision by adding more genetic and mensural data. Finally, I conclude my dissertation in chapter six.

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CHAPTER TWO

New species, diversity, systematics, and conservation assessment of the Puppet Toads of Sumatra (Anura: Bufonidae: *Sigalegalephrynus*)

This chapter were originally published as:

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ABSTRACT

Using a combination of morphological and molecular data we recognize three new species of Puppet Toad, *Sigalegalephrynus* Smart, Sarker, Arifin, Harvey, Sidik, Hamidy, Kurniawan & Smith, a recently described genus endemic to the highland forests of Sumatra, Indonesia. Phylogenetic analysis of mitochondrial DNA sequences recovered a monophyletic relationship among all Puppet Toads, with two distinct evolutionary clades, a northern and a southern. The northern clade includes *Sigalegalephrynus gayoluesensis*, and *S. burnitelongensis*, and the southern clade includes *S. harveyi*, *S. mandailinguensis*, and *S. minangkabauensis*. With the discovery of these three new species, *Sigalegalephrynus* contains more endemic species than any other genus of toad in Indonesia. We used maximum entropy, implemented in MaxEnt, to identify suitable habitats and occurrence probability of additional undescribed new species from the island. The most important predictors of *Sigalegalephrynus* distribution were elevation (64.5%) and land cover (7.11%). Based on the probability of presence, it is likely that there are many more species of the genus awaiting discovery in Sumatra. Our analysis, based on IUCN Red List of Threatened Species category and criteria, show that all of the five species of *Sigalegalephrynus* are in great risk of extinction and should be placed into the Endangered (EN) category of IUCN Red List.

Key words: GeoCAT, Indonesia, IUCN Red List, MaxEnt, Niche modeling, *Sigalegalephrynus burnitelongensis*, *Sigalegalephrynus gayoluesensis*, *Sigalegalephrynus harveyi*, *Sigalegalephrynus mandailinguensis*, *Sigalegalephrynus minangkabauensis*, Southeast Asia, Sunda Shelf

INTRODUCTION

Amphibians are a highly threatened and poorly-known animal group worldwide (Tapley *et al.* 2018), as is indicated by the many new species that have been described in recent years from numerous biodiversity hotspots. Unfortunately, these are facing extinction due to global spread of zoonotic disease (Rödder *et al.* 2009, Brito *et al.* 2011, O'Hanlon *et al.* 2018), habitat destruction and fragmentation (Margono *et al.* 2014, Harris *et al.* 2017), and anthropogenic climate change (Rödder *et al.* 2010, Bickford *et al.* 2010). Southeast Asia is a hotspot of amphibian diversity (Inger 1999), but still poorly understood (Brown & Stuart 2012, Chan & Grismer 2019, Coleman *et al.* 2019) and with many new species awaiting discovery (Bickford *et al.* 2010). Here, Sumatra is one of the least explored lands in terms of herpetological research (Harvey *et al.* 2017a). Extensive surveys are needed to understand its biodiversity (Brown & Stuart 2012) and to develop conservation strategies of this island (Bickford *et al.* 2010, Coleman *et al.* 2019).

The International Union for the Conservation of Nature (IUCN) Red List of Threatened Species is a widely-accepted index for assessing species extinction risks and an effective source for conservation planners (Lamoreux *et al.* 2003; Rondinini *et al.* 2013; Trull *et al.* 2017). Although there is a constant rate of discovery of amphibian species, an IUCN Red List status assessment accompanying those publications has declined from 2004 to 2015 (Tapley *et al.* 2018). Even though 24% of the amphibian species have been categorized as Data Deficient (DD) (Nori *et al.* 2018), about 80% do not have their up-to-date IUCN Red List status (Tapley *et al.* 2018) (many in Malaysia, Indonesia, Papua New Guinea, China). To help prioritize conservation for the newly discovered arboreal Puppet Toads, genus *Sigalegalephrynus* Smart, Sarker, Arifin, Harvey, Sidik, Hamidy, Kurniawan & Smith (2017), we describe three newly discovered populations as new species and provide IUCN Red List status assessment for all members of this Sumatran endemic genus.

The genus *Sigalgalephrynus* contains two species: *S. mandailinguensis*, from Gunung Sorikmerapi, Batang Gadis National Park, Sumatera Utara province, and *S. minangkabauensis*, from Gunung Kunyit, a peak in the Barisan Range of the province of Jambi (Smart *et al*. 2017). In 2015, we collected additional specimens of this genus, from the Burni Telon volcano and Highlands of Gayo Lues in the province of Aceh, and from Dempo volcano of the Sumatera Selatan province. Based on morphological characteristics, genetic divergence and advertisement calls, we recognize these populations as distinct species under the lineage-based Unified Species Concept of de Queiroz (2007), based on morphological characteristics, genetic divergence, and advertisement call. Herein we formally describe these three montane populations of Puppet Toads as new species, estimate their phylogenetic relationships, and discuss the distribution and conservation of the genus.

MATERIAL AND METHODS

Specimens of the new species described in this study were collected in July and August 2015. We strictly followed protocols approved by the UTA Institutional Animal Care and Use Committee (IACUC; number UTA IACUC A12.004) for collecting, handling and euthanizing specimens. We took photographs of live animals, and then pictures of dorsal, ventral and lateral aspects immediately after euthanasia. Specimens were fixed in 10% formalin, and then transferred to 70% alcohol for permanent storage. Prior to fixation, we took liver or muscle tissue samples and preserved them in 1.5 mL of cell lysis buffer solution (0.5 M Tris/0.25% EDTA/2.5% SDS, pH = 8.2). Finally, we deposited all specimens at Laboratory of Herpetology in the Museum Zoologicum Bogoriense (MZB), and the Amphibian and Reptile Diversity Research Center (ARDRC) of the University of Texas, Arlington (UTA). All other museum acronyms follow Sabaj Perez (2016).

Morphological data. Our morphological terminology is based on Matsui (1984), Duellman (2001), and Kok & Kalamandeen (2008). We used digital calipers or an ocular micrometer to measure each character to the nearest 0.1mm. We measured: 1) snout–vent length (SVL)—tip of snout to anterior margin of vent; 2) head length (HL)—posterior angle of jaw to tip of snout; 3) head width (HW)—ventrally at angles of jaw, excluding warts; 4) snout length (SNL)—anterior corner of eye to snout tip; 5) intercanthal distance (ICD)—distance between anterior edges of canthi; 6) internarial distance (IND)—distance between anterior ends of nares; 7) eye to naris distance (END)—distance from the anterior corner of eye to posterior border of naris; 8) naris to snout distance (NSD)—distance from anterior border of naris to the tip of snout; 9) interorbital distance (IOD)—minimal distance between upper eyelids; 10) eye length (EL)—horizontal distance from anterior to posterior junctions of upper and lower eyelids; 11) tympanum length (TML)—horizontal width of tympanum; 12) forearm length (FAL)—tip of elbow to proximal margin of outer metacarpal tubercle; 13) hand length (HAL)—proximal margin of metacarpal tubercle to Finger III tip; 14) thigh length (THL)—center of cloaca to distal surface of knee, appressed; 15) tibia length (TBL)—greatest length of tibia when positioning hind limb in a Z pattern; 16) tarsus length (TRL)—tibio-tarsal articulation to proximal margin of outer metatarsal tubercle; 17) foot length (FTL)—proximal margin of outer metatarsal tubercle to Toe IV tip; 18) outer metacarpal tubercle length (OMCL)—from the anterior to the posterior end of the outer metacarpal tubercle; 19) outer metacarpal tubercle width (OMCW)—greatest width of outer metacarpal tubercle measured perpendicularly to OMCL; 20) inner metacarpal tubercle length (IMCL)—from the anterior to the posterior end of the inner metacarpal tubercle; 21) inner metacarpal tubercle width (IMCW)—greatest width of outer metacarpal tubercle measured perpendicularly to IMCL; 22) inner metatarsal tubercle length (IMTL)—from the anterior to the posterior end of the inner metatarsal tubercle; 23) inner metatarsal tubercle width

(IMTW)—greatest width of inner metatarsal tubercle measured perpendicularly to IMTL; and 24) length of fingers (F1L–F4L)—from tip of fingers to first phalangeal-metacarpal joint; 25) length of toes (TlL–T5L)—from tip of fingers to first phalangeal-metatarsal joint; 26) width of third finger disc (F3PD)—at right angle to digital axis; 27) width of the proximal end of the penultimate phalanx of third finger (F3PB)—at right angle to digital axis. Along with mensural data, we took qualitative morphological characters (e. g. color) from each specimen. The webbing formulae follow Savage & Heyer (1967) as modified by Myers and Duellman (1982) and Savage & Heyer (1997). We used digital color photographs and followed Kok & Kalamandeen (2008) to describe color in life and other qualitative characteristics; these images are deposited at the University of Texas at Arlington digital image collection. All morphological mensural data were collected from adult specimens by a single observer (GCS).

Acoustic data analysis. Calls were recorded by using a Zoom H4n Handy Recorder® at a sampling rate of 44.1 kHz. For call analyses, we followed the terminology used by Duellman (1970). We performed call analyses by removing background noise with the sound-editing software Audacity v2.1.2 (Audacity Team 2016). To produce oscillograms and spectrograms we used RAVEN LITE 2.0.0 (Bioacoustics Research Program 2016). We calculated the time between notes, the time between pulses, fundamental frequency, and dominant frequency using SOUNDRULER version 0.9.6.0 (Gridi-Papp 2007).

Taxon sampling and DNA sequencing. To isolate genomic DNA we used Serapure beads following the Agencourt protocol (Beckman Coulter Co., Fort Collins, CO, USA) after Rohland & Reich (2012). We amplified one mitochondrial (16S) gene using the forward primer 16SH (5' CGC CTG TTT ATC AAA AAC AT 3') and the reverse primer 16SL (5' CCG GTC TGA ACT CAG ATC ACG T 3'), after Vences *et al.* (2005), using the thermocycler PCR protocol provided by Van Bocxlaer *et al.* (2009), and the Go Taq® Flexi

DNA polymerase (Promega Corporation, Madison, Wisconsin, USA), on a GeneAmp® PCR System 9700 (Applied BioSciences, Foster City, CA, USA). PCR success was visually assessed on a 1% agarose gel, and PCR products were purified with Serapure beads (following the Agencourt protocol, Beckman Coulter Co., Fort Collins, CO, USA). The Genomic Core Facility at the University of Texas at Arlington completed the sequencing reactions with an ABI PRISM 3100xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

We used 32 sequences for phylogenetic inference, including ten sequences from GenBank. All sequences generated for this project were submitted to GenBank (Table 1). Our taxon sampling included at least one representative from each extant Southeast Asian bufonid genus. We included one New World bufonid – American Toad (*Anaxyrus amaricanus*), and one hylid – Canyon Treefrog (*Dryophytes arenicolor)* as outgroups.

Sequence alignment and phylogeny inference. We assembled and cleaned raw gene fragment sequences using Sequencher v 5.3 (Gene Codes, Ann Arbor, MI, USA) and aligned the sequences using the Muscle (Edgar 2004) algorithm in MEGA (v6.0; Tamura *et al*. 2013). We used the Gblock server (Castresana 2000) to identify poorly resolved regions of the alignment, using a less stringent selection of blocks, which is more appropriate for short alignments (Castresana 2000; Talavera & Castresana 2007). We removed these poorly aligned regions from subsequent analyses.

To examine the phylogenetic relationships of *Sigalegalephrynus* we used maximum likelihood (ML) and Bayesian inference (BI) methods. PartitionFinder v1.10 (Lanfear *et al.* 2012) was used to determine the best partitioning schemes and respective nucleotide substitution models. We employed the corrected Akaike Information Criterion (AICc) model

to find selection parameters and the 'greedy' search algorithm for finding the best models for Bayesian analysis.

The GTR+I+G model was suggested for Bayesian analysis, but following Stamatakis (2006), we used GTR+G instead. We performed ML analysis employing a rapid bootstrapping algorithm using the program RAxML-HPC BlackBox (v8.2.10; Stamatakis 2014) on the CIPRES gateway server (Miller *et al.* 2010). We considered nodes to be strongly supported when having bootstrap value were above 70% (Hillis & Bull 1993).

We conducted Bayesian Markov chain Monte Carlo (MCMC) phylogenetic analyses using MrBayes (v3.2.3; Ronquist *et al.* 2012), employing two simultaneous runs of four MCMC analyses, consisting of one cold and three incrementally heated chains, with random trees for a total of 10×10^6 generations (sampling every 500 generations). We set the burn-in to the default value of 25%, hence discarding the initial 5000 generations. To examine stationarity, we used trace plots and ESS values (> 200) on TRACER v1.6 (Rambaut *et al.* 2014). We constructed a 50% majority consensus tree with estimates of Bayesian support using the remaining sampled trees and posterior probabilities (PP). We considered nodal support with PP values ≥ 0.95 as significant (Huelsenbeck & Rannala 2004; Mulcahy *et al.* 2011). We used FigTree (v1.4.2; Rambaut, 2012) for graphical visualization of the resulting ML and Bayesian trees.

Niche modeling and habitat suitability. Because there are many highland forests we could not explore during our Sumatran expedition, we used species distribution modeling techniques to identify suitable habitat for the most probable occurrence of additional new species of this genus. We used MaxEnt v.3.4.1 (Phillips *et al.* 2017), a maximum entropy model implementation, because it performs best among all other available species distribution modeling programs (Pearson *et al.* 2007; Tarkesh & Jetschke 2012, Remya *et al.* 2015).

Several studies (Galante *et al.* 2018, Pearson *et al.* 2007, Shcheglovitova & Anderson 2013) have shown that MaxEnt can have a biologically meaningful model performance with as low as five occurrence data points. For a very narrow-ranged species, this minimum number of occurrence data points can be as low as three (Proosdij *et al.* 2016) since MaxEnt is less sensitive to sample size (Wisz *et al.* 2008). *Sigalegalephrynus* species are micro-endemics (Smart *et al.* 2017) and occurrence data of these is limited, in fact we have no more than two useful occurrence data points for a single species. Thus, for finding suitable habitats for potential new populations or species, instead of modeling each species individually we collectively modeled our nine GPS data points for the five *Sigalegalephrynus* species.

For environmental variables, we used the 19 environmental variables representing seasonality, extremity, and annual trend available from the WorldClim v2.0 (http://worldclim.org/version2) datasets (Fick & Hijmans 2017), 30 arc seconds land cover data (http://www.diva-gis.org/gdata), and 30 arc-second digital elevation modeling data (DEM) from Southeast Asia (downloaded using ArcGIS online extension (ArcGIS v10.5; ESRI, Redlands, CA). Correcting for autocollinearity and excluding highly correlated variables is important in predicting future range shift of a species under climate change scenario (Braunisch *et al.* 2013). But, autocollinearity is not a problem for predicting the current range of a taxon (Braunisch *et al.* 2013, Brown *et al.* 2017).

Inclusion of all of the environmental variables do not affect the overall predictive quality of the MaxEnt model (Brown *et al.* 2017). On the other hand, exclusion of correlated environmental variables from the model needs a good understanding of the ecology and natural history of the species (Dufresnes *et al.* 2018; Spear *et al.* 2018). Also, the exclusion of environmental variables from the model could underperform and fail to predict the actual species occurrence data points too (Tan *et al.* 2017). Moreover, correcting for autocollinearity and deciding variable importance in modeling is not difficult for the machine learning

algorithm of MaxEnt (Elith *et al.* 2011; Shaney *et al.* 2017) which can be performed though jackknifing (Shaney *et al.* 2017). Many recent studies have used all of the 19 bioclimatic variables in species distribution modeling for predicting current distribution of species (e. g. for – arthropods: Perger *et al*, 2017, Bagheri *et al*. 2018; fish: Jarić *et al.* 2018; amphibians: Dufresnes *et al.* 2018, Neal *et al.* 2018; reptiles: Brothers & Lohman 2018, Shaney *et al.* 2017; birds: Berzaghi *et al.* 2018; mammals: Regmi *et al.* 2018; plants: Kodis *et al.* 2018, Karrenberg *et al.* 2018, Kim *et al.* 2018).

Given that multicollinearity is not a problem for predicting current distribution, we do not have a good understanding of the ecology and natural history of all the species of *Sigalegalephrynus* and aim of this modeling is not to predict future range shift under climate change scenario, we decided to include all 19 bioclimatic variables in our model following Spear *et al.* (2018). We jackknifed our model to assess best for predictor variables the distribution of *Sigalegalephrynus* (Merow *et al.* 2013, Brown 2014, Remya *et al.* 2015, Shaney *et al.* 2017). We used 75% of the data for training and 25% for testing following Hu *et al.* (2016). We used default regularization since it performs better in choosing reasonable predictors (Phillips *et al.* 2006). Four replicates were performed using subsample replication run type and maintaining 1000 iterations. We followed Hu and Jiang (2018) in using a 10^{th} percentile training threshold for creating a binary presence/absence map from the continuous suitability map output. We reported the area under the receiver operating characteristic (ROC) curve (AUC value). AUC value closer to 1.0 indicates high performance of the predictive model (Phillips and Dudík 2008, Walden-Schreiner *et al.* 2018), for each replication. We reported the average AUC value under the ROC curve of all replicates. Since sometimes AUC score itself under the ROC curve might be misleading (Lobo *et al.* 2008, Ramírez-Gíl *et al.* 2018), we assessed our model prediction performance by using functions available in NicheToolBox (http://shiny.conabio.gob.mx:3838/nichetoolb2/) (Osorio-

Olvera *et al*. 2018). We used similar parameters in NichToolBox that were used by Ramírez-Gíl *et al.* (2018) for assessing model prediction performance.

Geospatial conservation assessment. The extinction risks of all the species of *Sigalegalephrynus* were evaluated following the criteria and guidelines for the IUCN Red List Categories (IUCN Standards and Petitions Subcommittee, 2017). We also used the program GeoCAT (Bachman *et al.* 2011) for measuring Area of Occupancy (AOO) and Extent of Occupancy (EOO) by using IUCN recommended default values of the cell grid, and less stringent cell grid values (by doubling the IUCN default values).

RESULTS

Phylogenetic analyses. Our Bayesian and Maximum Likelihood analyses recovered monophyly and identical topologies for *Sigalegalephrynus,* with high support values (Fig. 1). Our phylogenetic analyses recovered two distinct clades, a northern clade that contains two undescribed new species, and a southern clade containing *S. mandailinguensis, S. minangkabauensis,* and a third undescribed species. Uncorrected pairwise interspecific distances in the mitochondrial ribosomal gene 16S rRNA fragment range from 4.0 to 10.4%, with significant divergence between the northern and southern clades, 8.2–10.4%, and moderate divergence within clades, 4.0–6.0% (Table 2).

Morphological characters also support a north/south clade division. Characters that define the southern clade include: (1) moderately mucronated (presence of rostral keel) snout in dorsal profile (vs truncated in northern clade), (2) body and limbs lanky (vs stocky in northern clade), (3) presence of distinct hourglass shape marking on dorsum (vs absent in northern clade), (4) body tubercles spinose (vs round in northern clade), (5) large tubercles on posterior of tympanum spinose (vs round in northern clade), (6) tympanum without elevated

annulus (vs elevated annulus in northern clade), and (7) lore with distinct white marking at sides (vs absent white bordering markings in northern clade).

Systematics

Sigalegalephrynus burnitelongensis

Figs. 2A–C, 4A, 5A, 6A

Holotype. Museum Zoologicum Bogoriense of Amphibian Collection, MZB.Amph.30413 (field number ENS 18884), an adult male. Collected from a stream of Gunung Burni Telong near Desa (Village) Rambune, Kecamatan (Subdistrict) Timang Gajah, Kabupaten (Regency) Bener Meriah, Province of Aceh, Indonesia. 4.76455ºN, 96.80138ºE, 1519 m a.s.l (Fig. 3). Collected by Goutam C. Sarker, Irvan Sidik, Syaripudin and Muhammad Ikhsan on 9 August 2015 at 00:30h.

Paratypes (2). The University of Texas at Arlington Amphibian collection numbers UTA A-65788 and UTA A-65492, adult males. Collected from near to the collection locality of the holotype, 4.76455ºN, 96.80138ºE, 1519 m a.s.l. (Fig. 3). Collected by Goutam C. Sarker, Irvan Sidik, Syaripudin and Muhammad Ikhsan on 8 August 2015 at 23:50h.

Referred specimens (33). All juveniles, UTA A−65493−509 (17), and MZB.Amph.26016−031 (16), same collection information as the types.

Etymology. The specific epithet is an adjective in Aceh language derived from Burni, meaning Mountain (*Gunung* in Indonesian) and Telong, meaning burning (*Bakar* in Indonesian), or in Sanskrit *Borni Təloŋ*, meaning “burning mountain”. This is the local name for the volcano that is the type-locality of this new species, and the Latin suffix *–ensis*, denoting place.

Suggested Common Name. Burning Mountain Puppet Toad, in English; *Kodok-wayang burnitelong*, in Indonesian.

Diagnosis. *Sigalegalephrynus burnitelongensis* can be diagnosed from its congeners by a unique combination of characters: (1) small-size (males 21.73–23.06 mm SVL); (2) lacking parotoid glands; (3) tympanum visible, with elevated annulus not encircled by sharply raised spinose tubercles; (4) naris closer to tip of snout than to eye; eye-naris distance 6.3% (8.3%, 6.9%) of SVL; naris-snout distance 1.1 % (1.2% 1%) of SVL, (5) fingertips truncated but not expanded (except finger I); (6) tips of toe I, II and III rounded, truncated but not expanded on toe IV and V; (7) rudimentary webbing in hands, moderate in feet; (8) dorsum brown without any marking; (9) medial dorsal dark band absent; (10) lacking alternate dark brown and white markings on upper lip, or not prominent; (11) flanks lacking stroke of different color; (12) dorsum lightly tuberculate, tubercles round; (13) venter pinkish–yellow, without maculation and uniformly tuberculate, (14) interocular distance 44% (43%) of head width; (15) nuptial pads dark brown, with black–tipped spicules; (16) finger IV tip not reaching distal phalangeal articulation of finger III (when adpressed); (17) inner metacarpal tubercle ¾ of outer metacarpal tubercle in length.

Description of holotype and variation of paratypes (in parenthesis). Body moderately robust; head slightly longer than wide, HL/HW = 1.03 (1.10, 1.02); head length 32% (32%, 31%) of SVL; head width 31% (29%, 31%) of SVL; snout length 11% (10, 11%) of SVL; *canthus rostralis* concave; loreal area slightly tuberculate and concave; eye length 10% (9%, 10%) of SVL; pupil circular; snout truncate in dorsal view and protruding (slightly sloping back towards mouth) in lateral view; tympanum round with distinct annulus; interorbital space flat; cranial crests absent; no teeth in jaws; tongue tip oval shaped and longer than wide; skin of dorsal surfaces slightly rough to finely shagreen, with few large, scattered, round tubercles; most tubercles small, almost without keratinization; no dorsolateral,

paravertebral, or occipital folds; skin on venter smoother, with very small and round tubercles; circumcloacal region golden yellow.

Arms robust, with moderately developed axillary membrane; forearm length 27% (27%, 26%) of SVL; hand length 27% (24%, 26%) of SVL; relative length of fingers: I < II < IV < III; fingers bearing large expanded pads; webbing formula for hand: I11/2 - 2II11/2 - 23/4III23/4 - 22/3IV (I1 - 1II3 - 23/4III 21/2 - 2IV); skin of forearm with tubercles; finger I with elongate inner metacarpal tubercle, smaller than the outer metacarpal tubercle; each finger with one poorly developed round subarticular tubercle; nuptial pads brownish-dark, glandular, and dorsomedially extended with black keratinized spicules present at the base of finger I.

Thigh length 44% (43%, 43%) of SVL; tibia length 41% (41%, 40%) of SVL; tarsal length 21% (21%, 24%) of SVL; foot length 41% (37%, 41%) of SVL; relative lengths of toes - I< II< III<V< IV; toes bearing large pads; feet with moderate webbing (Fig. 6A), webbing formula for the feet: I0 – 11/2II0 –2III11/2 – 3IV3 – 2V (I1 – 11/2II2 – 2III3 – 3IV2 –11/2V); heels without tubercles; inner metatarsal tubercle moderately developed and elongate; outer metatarsal tubercle distinct; one moderate subarticular tubercle present at the base of first phalanx on each toe; toes with toe pads.

Measurements (in mm). Holotype followed by paratypes in parenthesis. Finger III of right hand of paratype deformed, finger measurements of this specimen taken on left hand. SVL 22.18 (21.73, 23.06); HL 7.06 (6.96, 7.20); HW 6.84 (6.31, 7.04); SNL 2.40 (2.20, 2.45); ICD 3.70 (3.50, 3.80); IND 1.89 (1.91, 1.93); END 1.4 (1.80, 1.6); NSD 0.25 (0.26, 0.23); IOD 3.00 (3.10, 3.00); EL 2.20 (2.00, 2.25); TML 1.4 (1.45, 1.2); FAL 6.04 (5.90, 6.10); HAL 6.00 (5.30, 6.00); THL 9.70 (9.28, 9.82); TBL 9.09 (8.97, 9.24); TRL 4.55 (4.50, 5.51); FTL 9.00 (7.98, 9.34); OMCL 1.00 (1.00, 1.00); OMCW 1.00 (1.00, 1.00); IMCL 0.55

(0.50, 0.50); IMCW 0.75 (0.75, 0.75); IMTL 1.5(1.00, 0.90); IMTW 1.0(0.70, 0.80); F1L 0.80 (1.00, 1.00); F2L 1.60 (2.00, 2.00); F3L 3.15 (3.50, 3.50); F4L 2.10 (2.50, 2.50); T1L 1.00 (1.00, 1.00); T2L (1.40, 1.40); T3L 1.80 (2.00, 2.00); T4L 5.00 (4.00, 4.60); T5L 3.50 (3.0, 3.00); F3PD 0.90 (0.80, 1.00); F3PB 0.80 (0.60, 0.75).

Color of holotype in life. Adult male holotype (Figs. 2A, 2B, 2C): dorsum predominantly light brown, lacking distinct markings; flanks brown, lacking oblique stripes; infraorbital part of maxilla with light-brown marking; lore light brown, with small dark-brown spot between orbit and naris; dorsum of limbs brown, lacking distinctive crossbar markings; moderately large white tubercles at posterior mandibular articulation; abdominal surface pink, with many yellow blotches; gular region, clavicular, and ventral surface of limbs pink, without yellow blotches; tips of fingers and toes blackish, with golden yellow blotches; iris golden yellow, with heavy black reticulations.

Color of holotype in preservative. Differing slightly from that in life, pinkish coloration turned grey, and venter has turned whitish grey.

Comparisons. *Sigalegalephrynus burnitelongensis* is restricted to Gunung Burni Telong, a volcano in Bener Meriah regency, Sumatra, and does not exist in sympatry with any other congeners. *Sigalegalephrynus burnitelongensis* can be easily distinguished from all other congeners (including *S. gayoluesensis* from Gayo Lues Regency) by the lack of crossbar markings on the dorsal surface of the limbs. It differs from *S. mandailinguensis, S. minangkabauensis* and *S. harveyi* by its truncate (vs. mucronate) shaped snout in dorsal profile, stocky limbs (vs. lanky) smooth tubercles (vs. warty with sharp tips), and lacking an hourglass mark on the dorsum (vs. hourglass present).

Distribution and natural history. *Sigalegalephrynus burnitelongensis* is known only from forest patches associated to small streams and surrounded by coffee plantations, at

Gunung Burni Telong, near the village of Rambune in the province of Aceh, from 1519 m a.s.l. (Fig. 3). The holotype and paratype were found sitting on small leaves of shrubs 20 cm above ground. The holotype weighed 0.76 g, and the paratype 0.69 g. The smallest juvenile collected (UTA A-65505) was 9.6 mm in SVL and 0.06 g in weight.

Sigalegalephrynus gayoluesensis

Figs. 2D–F, 4B, 5B, 6B

Holotype. Museum Zoologicum Bogoriense of Amphibian Collection, MZB.Amph.30411 (field number ENS 19527). An adult male from above the Desa (Village) Kenyaran Pantan Cuaca, Kabupaten (Regency) Gayo Lues, Provinsi Aceh, Indonesia, 4.22588ºN, 97.18915ºE, 1850 m. a.s.l. (Fig. 3). Collected by Elijah Wostl, Ahmad Muammar Khadafi, and Syaripudin on 9 August 2015 at 21:20h.

Paratypes (3). The University of Texas at Arlington Amphibian collection number UTA A-65490, Museum Zoologicum Bogoriense of Amphibian Collections, MZB.Amph.26035, adult males; MZB.Amph.26037, adult female. Collected from near to the collection locality of the holotype, 4.22580ºN, 97.1886º1E, 1844 m. a.s.l. (Fig. 3). Collected by Elijah Wostl, Ahmad Muammar Khadafi, and Syaripudin on 9 August 2015 at 21:05h.

Referred specimens (8). Collection data similar to that of the types with the types. UTA A-65488−489, 65789 (subadult and two juveniles, respectively, 1827 m. a.s.l., 4.2239ºN, 97.18718ºE); 65790 (subadult, 1826 m. a.s.l., 4.22487ºN, 97.18769ºE); and MZB.Amph. 26032 (juvenile, 1827 m. a.s.l., 4.22357ºN, 97.186551º E); 26033 (juvenile,1827 m. a.s.l., 4.2239ºN, 97.18718ºE); 26034, 26036 (two juveniles,1826 m. a.s.l., 4.22487ºN, 97.18769ºE).

Etymology. The specific epithet refers to the Gayo Lues Highlands, where this new species was found.

Suggested Common name. Gayo Lues Highland's Puppet Toad; Indonesia name: Kodok-wayang gayolues

Diagnosis. *Sigalegalephrynus gayoluesensis* can be identified from its congeners by a unique combination of characters: (1) medium-size (adult males 25.65–26.49 mm SVL); (2) lacking parotoid glands; (3) tympanum visible, with elevated annulus, and not encircled by sharply raised spinose tubercles; (4) naris closer to tip of snout than to eye; eye-naris distance 6.4.0% (7%) of SVL; naris-snout distance 1% (1.9%) of SVL; (5) fingertips truncated and expanded (except finger I); (6) tips of toe I, II and III are rounded; tips of toes IV and V truncated but not expanded; (7) rudimentary webbing in hands, moderate in feet; (8) adult male dorsal coloration dark brown, with prominent whitish diamond shaped suprascapular marking; (9) dorsum lacking medial dark band; (10) upper lip with prominent alternating dark brown and white marks; (11) flanks with stroke of dark brown (demarcated by thin white lines on top and bottom), extending from orbit to inguinal area; (12) dorsal surface lightly tuberculate, with round tubercles; (13) venter pinkish–white, with black maculation; (14) interocular distance 43% (44%) of head width; (15) nuptial pads dark brown, with black–tipped spicules; (16) finger IV tip extending beyond distal (terminal) phalangeal articulation of finger III (when adpressed); (17) inner metacarpal tubercle ¾ length to outer metacarpal tubercle.

Description of holotype and variation of paratypes (in parenthesis). Body moderately robust; head longer than wide, HL/HW =1.14 (1.11, 1.07, 1.02); head length 33% (34%, 31%, 34%) of SVL; head width 29% (31%, 29%, 33%) of SVL; snout length 10% (10%, 10%, 11%) of SVL; *canthus rostralis* concave; loreal area without tubercles, concave; eye

length 10% (12%, 10%, 9%) of SVL; pupil circular; snout truncate in dorsal view, protruding in lateral view, sloping back towards mouth; tympanum distinct and rounded, with annulus, but not surrounded by large tubercles; interorbital space flat; cranial crests absent; no teeth in jaws; tongue tip oval shaped and longer than wide; skin of dorsum finely shagreened, with few large and scattered tubercles; tubercles rounded, without keratinization; no dorsolateral, paravertebral, or occipital folds; skin on venter smooth with anastomosis; circumcloacal region is golden yellow.

Arms robust; forearm length 31% (33%, 25%, 26%) of SVL; hand length 30% (31%, 26%, 27%) of SVL; relative length of Finger - I<II<IV<III; fingertips truncated and dilated; hands rudimentary webbed, hand webbing formula: I0 – 11/2II1 - 21/2III21/2 - 21/3IV (I[0] – [12/3–2]II[1–11/2] – [2–21/2]III[2–21/2] – [2–21/2]IV); skin of forearm with moderately developed tubercles; finger I with moderately developed inner metacarpal tubercle, smaller than the outer metacarpal tubercle; each finger with one poorly developed round subarticular tubercle; nuptial pads brownish-dark, glandular, dorsomedially extended; spicules of nuptial pads with black keratinized spicules.

Thigh length 45% (44%, 44%, 44%) of SVL; tibia length 40% (43%, 39%, 41%) of SVL; tarsal length 25% (25%, 21%, 20%) of SVL; foot length 41% (42%, 42%, 39%) of SVL; relative lengths of toes - I< II< III<V<IV; toes bearing large pads; feet with moderate webbing (Fig. 6B), webbing formula for the feet: I0 - 1/2II0 - 1III1 - 21/2IV23/4 - 2V (I[0] – [0–1/2]II[0] – [1]III[0–1/2] – [21/2]IV[21/2] - [12/3–2]V); heels without tubercles; inner and outer metatarsal tubercle moderately developed and elongate.

Measurements (in mm). Holotype followed by paratype in parenthesis: SVL 26.49 (25.65, 26.07, 27.36); HL 8.72 (8.84, 8.06, 9.20); HW 7.67 (7.98, 7.53, 9.0); SNL 2.75 (2.5, 2.55, 3.0); ICD 4.30 (4.50, 4.50, 4.56); IND 2.20 (1.80, 2.0, 2.0); END 1.7 (1.8, 1.55, 2.12);

NSD 0.25 (0.5, 0.5, 0.7); IOD 3.0 (3.5, 3.5, 4.0); EL 2.70 (3.0, 2.55, 2.55); TML 1.50 (1.55, 1.6, 1.47); FAL 8.24 (8.40, 6.5, 6.5); HAL 7.89 (7.86, 6.67, 7.5); THL 11.83 (11.34, 11.53, 12.07); TBL 10.68 (10.95, 10.29, 11.23); TRL 6.63 (6.48, 5.50, 5.50); FTL 10.83 (10.86, 10.90, 10.80); OMCL 1.0 (1.0, 10, 1.0); OMCW 1.0 (1.0, 1.0 1.0, 1.0); IMCL 0.75 (0.75, 0.65, 0.60); IMCW 0.50 (0.50, 0.50); IMTL 1.0 (1.0, 1.0, 1.0); IMTW 1.0 (1.0, 1.0, 1.0); F1L 1.5 (1.5, 1.5, 1.5); F2L 2.30 (2.25, 2.35 2.45); F3L 4.0(3.5, 3.5, 3.2); F4L 3.5 (3.0, 2.6, 2.55); T1L 2.0 (1.5, 1.5, 1.6); T2L 2.5 (2.0, 2.0, 2.0); T3L 3.2 (3.0, 3.3, 2.5); T4L 5.5 (5.0, 5.0, 4.5); T5L 4.0 (3.5, 3.5, 3.3); F3PD 1.25 (1.2, 1.0, 1.3); F3PB 1.0 (1.0, 0.9, 1.0).

Color of holotype in life. (Figs. 2D, 2E, 2F). Dorsum predominantly brown, with suprascapular dark brown diamond-shaped marking encircled by light brown; flanks with alternate wide dark brown and narrow white stripes; wide whitish light-brown spot below eye; lore dark brown, with small light brown spot adjacent to anterior of orbit; iris golden yellow, heavily reticulated; dorsum of limbs dark brown, with dark-brown crossbars; large white tubercles present at point of posterior mandibular articulation; abdominal surface pink, with dark brown maculation; throat pinkish, with no maculation; underside of limbs pink, with dark brown maculation; iris golden yellow, with black reticulations.

Color of holotype in preservative. In alcohol, pinkish coloration turned grey and venter whitish grey, maculated with dark brown blotches.

Advertisement call. The call of the male holotype was recorded in the field and before collection. Ambient temperature at the time of recording was 17.2 ºC. The call is composed of 179 highly modulated notes given 0.245 seconds apart, on average (range, 0.140–0.907 seconds, SD ± 0.148 seconds). On average, each note is 0.049 seconds (range, 0.24–0.93, SD ± 0.18 seconds) in length and is composed of one distinct pulse. The average fundamental and dominant frequencies of the vocalization are 2474.361 (range, 2368.652–2627.051 Hz,

SD ± 85.86 Hz) Hz and 4948.722 Hz (range, 4737.305–5254.102 Hz, SD ±171.7309 Hz) respectively (Fig. 7).

Comparisons. *Sigalegalephrynus gayoluesensis* is likely restricted to the mountains of the Gayo Lues Regency of Aceh, Sumatera, and does not exist in sympatry with any other congener. *Sigalegalephrynus gayoluesensis* can be easily distinguished from *S. mandailinguensis*, *S. minangkabauensis* and *S. harveyi* by its smooth tubercles on the body (vs. sharp-tipped warty tubercles) and a diamond shaped marking on the dorsum (vs. hourglass in *S. mandailinguensis*, *S. minangkabauensis* and *S. harveyi*, no hourglass or diamond shape mark in *S. burnitelongensis*). *Sigalegalephrynus gayoluesensis* can also be distinguished from *S. burnitelongensis* by its black anastomotic maculated throat and abdomen (vs immaculate throat and abdomen).

Acoustic data is limited for *Sigalegalephrynus* species, the call of the holotype of *S. gayoluesensis*, differs from that of *S. mandailinguensis* in duration (46.448 s vs 17.27 s), total number of notes (179 vs 62), notes per second (4 vs 6–7), average note length (0.49 s vs 0.029 s), average pause length between notes (0.245 s vs 0.012 s), and dominant frequency (4948.722 Hz vs 3400 Hz) (Fig 5).

Distribution and natural history. *Sigalegalephrynus gayoluesensis* is known only from rain forest flanking a stream adjacent to the Takengon-Blangkejeren road above the village Kenyaran Pantan Cuaca, in the Gayo Lues Regency of the province of Aceh, between 1787 and 1796 m a.s.l. (Fig. 3). Both the holotype and paratype were found calling on broad smooth leaves, at 1.6 m and 3.8 m above ground, respectively. The call of the holotype was recorded. The call sounded similar to that of *S. mandailinguensis* at the time of recording. Both the holotype and paratype weighed 1.27 g. Our smallest juvenile of this species (UTA A-65789) was less than 1 cm (SVL 8.0 mm) in SVL and weighed 0.05 g.

Sigalegalephrynus harveyi

Figs. 2M–O, 4E, 5E, 6E

Holotype. Museum Zoologicum Bogoriense of Amphibian Collection, MZB.Amph.30412 (field number ENS 18377). An adult male from Gunung Dempo above the Desa (Village) Kampung Empat, Kabupaten (Regency) Pagar Alam, Provinsi Sumatera Selatan, Sumatra, Indonesia, 4.040980ºS, 103.1481ºE, 1826 m a.s.l. (in all cases, datum = WGS84) (Fig. 3). Collected by Michael B. Harvey, Farits Alhadi, and Panupong Thammachoti on 8 July 2015, at 21:35h.

Paratype. The University of Texas at Arlington Amphibian Collection UTA A-65474, an adult male. Collected from near to the collection locality of the holotype, 4.03923ºS, and 103.1473ºE, 1878 m a.s.l (Fig 3). Collected by Michael B. Harvey, Panupong Thammachoti, and Gilang Pradana on 10 July 2015, at 20:55h.

Etymology. The specific epithet is a patronym in honor of Michael B. Harvey, one of the collectors of this new species, a friend, an outstanding herpetologist, and the co-Principal Investigator of the National Science Foundation (NSF) project that has contributed this and a significant number of other papers on the herpetofauna of Sumatra.

Suggested Common Name. Harvey's Puppet Toad, in English; *Kodok-wayang Harvey*, in Indonesian.

Diagnosis. *Sigalegalephrynus harveyi* can be identified from its congeners by a unique combination of characters: (1) medium-sized (adult males 26.36–28.09 mm SVL) *Sigalegalephrynus*; (2) lacking parotoid glands; (3) tympanum visible, with elevated annulus

encircled with sharply raised spinose tubercles; (4) naris closer to tip of snout than to eye; eye-naris distance 8.0% (9.3%) of SVL; naris-snout distance 2.8 % (2.1%) of SVL; (5) fingertips truncated (except finger I), but not expanded; (6) tips of toes I, II, III and V rounded, toe IV tip truncated, but not expanded; (7) webbing rudimentary in hands, moderate in feet; (8) dorsal coloration in adult males light brown, with a prominent hourglass shaped marking; (9) dorsum, lacking medial dark band; (10) prominent alternate dark brown and white marks on upper lip; (11) flanks with dark brown strokes (demarcated by thin white lines on top and bottom), extending from orbit to inguinal area; (12) dorsal surface very lightly tuberculate, with white tipped spinose tubercles; (13) venter golden–yellow, without dark maculation; (14) interocular distance 48% (52%) of head width; (15) nuptial pads white, with white–tipped spicules; (16) finger IV tip touches distal phalangeal articulation of finger III (when adpressed); (17) inner metacarpal tubercle equal in length to outer metacarpal tubercle.

Description of holotype and variation in paratype (in parenthesis). Body slender; head longer than wide, HL/HW 1.11 (1.14); head length 30% (33%) of SVL; head width 27.0% (29%) of SVL; snout length 13% (14%) of SVL; *canthus rostralis* concave; loreal area smooth and concave; eye length 10% (10%) of SVL; pupil circular; snout slightly sloping back, towards mouth; snout mucronate, with prominent median keel, protruding in lateral view; tympanum distinct, rounded, with moderately developed annulus; interorbital space flat; cranial crests absent; jaws toothless; tongue tip oval shaped and longer than wide; dorsal skin tuberculate and rough, with mostly small and white tipped tubercles, lacking black keratinization; tympanum with elevated and distinct annulus, circled by large tubercles; no dorsolateral, paravertebral, or occipital folds; throat golden yellow; venter pinkish and golden-yellow, areolate in texture; circumcloacal region brownish yellow.

Arms lanky, with poorly developed axillary membranes; forearm length 28% (28%) of SVL; hand length 27% (28%) of SVL; relative length of fingers I < II < IV < III; fingertips truncated but not expanded; fingers bearing moderate pads; hands rudimentary webbed, hand webbing formula: I13/4 - 2II13/4 - 3III3 - 3IV (I13/4 - 2II13/4 - 3III3 - 3IV); flanks and dorsal surface of forearms tuberculate; inner metacarpal tubercle elongate, as large as outer metacarpal tubercle; fingertips truncated but not dilated; finger I and II with moderately developed basal round subarticular tubercles; subarticular tubercle on finger I is equal in size to the inner metacarpal tubercle; fingers III and IV with poorly developed basal round subarticular tubercles; nuptial pads white, glandular, dorsomedially extended; spicules of nuptial pads white tipped.

Thigh length 41% (43%) of SVL; tibia length 38% (41%) of SVL; tarsal length 24% (23%) of SVL; foot length 42% (41%) of SVL; relative lengths of toes I < II < III< V< IV; feet moderately webbed (Fig. 6E), foot webbing formula: I0 - 2II0 - 2III13/4 - 3IV3 - 2V (I0 - 1II0 - 2III11/2 - 3IV3 - 2V); heels without tubercles; inner metatarsal tubercles oval and well developed; inner metatarsal tubercle round and larger than the outer metatarsal tubercle.

Measurements (in mm). Holotype followed by paratype in parentheses: SVL 26.36 (28.09); HL 8.0 (9.41); HW 7.18 (8.25); SNL 3.4 (3.8); ICD 4.2 (4.5); IND 2.0 (2.60); END 2.6 (12.1); NSD 0.06 (0.75); IOD 3.7 (4.0); EL 2.55 (2.70); TML 1.3 (2.1); FAL 7.25 (7.95); HAL 7.09 (7.80); THL 10.72 (11.96); TBL 10.11 (11.45); TRL 6.20 (6.41); FTL 10.96 (11.62); OMCL 1.0 (1.0); OMCW 1.0 (1.0); IMCL 1.0 (1.0); IMCW 0.38 (0.50); IMTL 1 (1); IMTW 1 (1); F1L 1.5 (1.8) ; F2L 2.25 (2.30); F3L 3.65 (3.98); F4L 2.75 (3.40) ; T1L 1.0 (1.5); T2L 1.5 (2); T3L 3.0 (3.0); T4L 5.0 (5.5); T5L 3.5 (4.0).; F3PD 0.75 (1.0); F3PB 0.75 (1.0).

Color of holotype in life. Adult male holotype (Figs. 2M, 2N, 2O): dorsum predominantly brown, with an hourglass marking with whitish brown halo; iris brownish-yellow; flanks with alternate wide dark-brown and thin white oblique stripes, extending from post-ocular to inguinal areas; a very dark brown triangular blotch below anterior half of eye, with thin posterior white border that extends posteriorly on subocular rim; loreal region brown; dorsum of limbs darker than body dorsum, humeral and femoral segments without crossbars, distal segments with crossbars; area of posterior mandibular articulation with a whitish-yellow spot; lower flanks, inguinal, and circumcloacal regions golden-yellow; underside of body and head yellowish, with heavily melanized chest; ventral limb surfaces brown-salmon color; finger and toe tips pale salmon color, not melanized; iris bronze with black reticulations.

Color of holotype in preservative. Differing slightly from that in life, specimens have lost the golden yellow and pinkish coloration, which has turned grey.

Comparisons. *Sigalegalephrynus harveyi* differs from all congeners by the combination of possessing truncated but not expanded fingertips (except finger I) (vs truncated and highly expanded in *S. gayoluesensis* and *S. burnitelongensis*; truncated and moderately expanded in *S. mandailinguensis*; round in *S. minankabauensis*), and white tipped tubercles on the body (vs black tipped in *S. mandailinguensis* and *S. minangkabauensis*). Additionally, *Sigalegalephrynus harveyi* has a prominent hourglass shaped marking on the dorsum (vs missing in adult males of *S. burnitelongensis*), white-spiculed nuptial pads in adult males (vs black or dark brown tipped in *S. mandailinguensis, S. gayoluesensis, S. burnitelongensis*, unknown in *S. minangkabauensis*), an indistinct white loreal spot (vs very distinct in *S. mandailinguensis* and *S. minangkabauensis*, absent in *S. gayoluesensis* and *S. burnitelongensis*), inner and outer metacarpal tubercles of equal size (vs inner metacarpal tubercle larger in *S. mandailinguensis* and *S. minangkabauensis*, and smaller in *S.*

gayoluesensis and *S. burnitelongensis*, with respect to outer metacarpal tubercle), and Finger IV tip (when adpressed) not touching the terminal (distal) phalangeal articulation of Finger III (vs touching in *S. mandailinguensis S. minankabauensis*, and going beyond the articulation in *S. gayoluesensis*) (Fig. 6E).

Distribution and natural history. *Sigalegalephrynus harveyi* is only known from montane cloud-forest on the south-eastern slopes of Gunung Dempo, from 1826 and 1878 m a.s.l. (Fig. 3), and does not exist sympatrically with any other congener. The holotype was found calling on a leaf about 2 m above ground. Call was not recorded. The paratype was inactive on a leaf, 10 cm above ground. The holotype was not weighed, the paratype was 1.09 g.

Key to the species of *Sigalegalephrynus*

1 Adult males have a stout body with stocky limbs, and dorsum with a white diamond shaped mark or unmarked (Figs. 4A–B); snout truncated in dorsal profile, and tympanic annulus well developed and covered with sharply raised tubercles (Figs. 5A–B) 2

- Adult males and juveniles with a gracile body and lanky limbs, and dorsum with an hourglass shaped mark (Figs. 4C–E); snout moderately mucronated in dorsal profile, and tympanic annulus not covered by sharply raised tubercles (Figs. 5C–E) 3

2 Adult males >24 mm in SVL, a white diamond shaped mark present on dorso-scapular region, and venter maculated in adult males (Fig. 4B); subarticular tubercle of finger I as wide as width of inner metacarpal tubercle, tip of finger IV extending beyond distal phalangeal articulation of finger III, when addpressed (Fig. 6B) *S. gayoluesensis*

- Adult males <24 mm in SVL, dorsum without marking, and venter without maculation (Fig. 4A); inner metacarpal tubercle wider than long, subarticular tubercle of

finger I as wide as inner metacarpal tubercle, and tip of finger III not extending beyond distal phalangeal articulation of finger III, when addpressed (Fig. 6A) *S. burnitelongensis*

3 Venter in adult males maculated or blotched (Figs. 4C, 4E); webbing between toes I and II not complete (Figs. 6C, 6E); posterior mandibular articulation with a white spot on each side, and post-tympanic region with black and white large tubercles (Figs. 5C, 5E)

4

- Venter in juveniles yellow with black blotches (Fig. 4D); webbing between toes I and II complete (Fig. 6D); posterior mandibular articulation without a white spot on each side, and post-tympanic region with only white large tubercles; fingertips rounded (Fig. 5D) *S. minangkabauensis*

4 Adult males >30 mm in SVL, venter in adult males maculated and anastomotic, and tubercles on body with dark brown or black keratinized tips (Fig. 4C); nuptial pads in adult males with black-tipped spicules (Fig. 5C); finger tips truncated and expanded (Fig. 6C) *S. mandailinguensis*

- Adult males <30 mm in SVL, venter in adult males not maculated and anastomotic, but slightly spotted, and tubercles on body round and white-tipped (Fig. 4E); nuptial pads in adult males with white-tipped spicules (Fig. 5E); fingertips truncated but not expanded (Fig. 6E) *S. harveyi*

Niche modeling, distribution, and conservation status of *Sigalegalephrynus*

All known Puppet Toads are found in the highland forests of Sumatra between 1200 and 1900 m a.s.l. (Fig. 8A). Our logistic output for habitat suitability distribution of *Sigalegalephrynus* species had a very high success rate. Our average test AUC score for the

replicate runs was 0.945, with a standard deviation of 0.027. Jackknife variable contribution test revealed that among the variables used for the modeling, elevation contributed most significantly (64.5%) to the habitat suitability, followed by land cover (7.1%). Our model identified many additional isolated mountain tops as suitable habitat for *Sigalegalephrynus* species (Fig. 8B). The total area of all suitable habitat in Sumatra equaled 445 km^2 which is only 1.78% of the total montane forests of Sumatra (Margono *et al.* 2014). All the suitable habitats are highland forests above 1200 meters in elevation (Fig. 8C).

Our GeoCAT analysis revealed that extent of occurrence (EOO) of each species of *Sigalegalephrynus* is less than 1 km^2 and area of occupancy (AOO) is between 4 and 8 km^2, suggesting all species are Critically Endangered (CR), based on both EOO and AOO status. Doubling the IUCN default grid values in GeoCAT, we found that area of occupancy (EOO) is between 4 and 36 km^2 suggesting Critically Endangered (CR) category but the AOO status suggesting Endangered (EN) category for all of the *Sigalegalephrynus* species (Table 3).

DISCUSSION

Sigalegalephrynus is one of the four enigmatic bufonid genera in South and Southeast Asia and one of the key components to understand biodiversity of the region (Chan and Grismer 2019) that needs more studies. This is only the second study on the genus *Sigalegalephrynus*, and with this discovery of three new species, the genus *Sigalegalephrynus* becomes the most diverse endemic bufonid in Indonesia. These are micro-endemic frogs that are restricted to the mountain tops of the Barisan Range of Sumatra. The inter-specific divergence of the mitochondrial 16S rRNA gene between *S. harveyi* and *S. mandailinguensis* is 4.2%, which exceeds the conventional threshold values considered by many anuran phylogeny studies for recognizing distinct species—3% in Fouquet *et al.* (2007) and Vieites *et al.* (2009), 1.6% in

Bell (2016), 2–2.5% in Zimkus *et al.* (2017), and 2.3% in Tapley *et al.* (2017). Our phylogenetic data shows a deep divergence (8.2%-10.4%) (Table 2) between the clades north and south of Lake Toba. A similar divergence pattern is also identified in the highland agamid lizards of the genus *Dendrogama* (Harvey *et al.* 2017b) and in the frogs of the genus *Rhacophorus* (O'Connell *et al.* 2018).

Crow (2005) suggested continuous volcanic activity through the Bukit Barisan mountain range of Sumatra until the late Miocene, with reduced volcanic eruptions afterward, and according to Setyaningsih *et al.* (2018) that active volcanism greatly affected the ecosystem and biodiversity of the region. Lohman *et al.* (2011) suggested that the inundation of lowlands due to rising of sea level during the Pliocene and Pleistocene, formed isolated highland refugia throughout the Barisan Range, having a great influence on Sumatran biodiversity. Thus, the deep divergence between the northern and southern clades might be the result of ancient volcanic eruptions in the late Miocene. Further diversification within the group might be a result of more recent volcanic orogeny and glacial cycles and related sea level fluctuations during the late Pliocene and early Pleistocene.

Proper estimation of biodiversity is the key component for conservation. Conservation and management efforts become more challenging where the vast majority of species of an area—for example, Sumatra—is yet to be discovered (Grismer *et al.* 2013). New species are most likely to be found on each isolated mountaintop within the distribution of a genus containing species generally small sized, secretive, and with special habitat requirements (Grismer 2006). Given the arboreal nature with close ties to the stream systems of montane forests of Sumatra, it is highly likely that there are many more *Sigalegalephrynus* species awaiting discovery in unexplored mountain tops in Sumatra. With a high average AUC test score for replicates (0.945±0.027) our MaxEnt output presents a high level of accuracy in model prediction, and our partial model validation test for ROC was also significant

($P<0.001$). Since the MaxEnt modeling output with 10^{th} percentile threshold rule for suitable habitat (Hu & Jiang 2018) recovered at least 17 mountains with montane forests that are more than 1100 m a.s.l., isolated, and forested (Fig.8B and 8C), it would not be surprising to find as many as 12 more new populations of *Sigalegalephrynus* in Sumatra. Given the microendemicity observed in *Sigalegalephrynus*, all of these could represent distinct species. Despite recent discoveries of other anurans in Sumatra (Teynie *et al.* 2010, Matsui *et al.* 2012, Streicher *et al*. 2014, Hamidy & Kurniati 2015, Wostl *et al.* 2017, Arifin *et al*. 2018), anuran diversity in the island still remains significantly underestimated (Stuart *et al*. 2006, Inger *et al.* 2009, Arifin *et al*. 2018). Intensive surveying for new amphibian species on individual mountains of Sumatra is imperative for documenting underrepresented anuran diversity.

About 32% of amphibian species are threatened globally, the highest percentage among all threatened quadruped vertebrate classes (IUCN 2017). Southeast Asian amphibians are no exception to the threats, and they are facing a grave conservation crisis (Rowley *et al.* 2009, Coleman *et al.* 2019), compounded day by day by global climate change (Bickford *et al.* 2010, Kusrini *et al.* 2017), overexploitation (Natusch & Lyons 2012), habitat loss and deforestation (Daszak *et al.* 2003), chytrid fungus infestation (Kusrini *et al.* 2017, Hamidy *et al.* 2018), and lack of information on conservation status (Tapley *et al.* 2018). In Indonesia, 41.4% of amphibians are endemic (Sodhi *et al.* 2004), and 65.6% of these are threatened (IUCN 2017). The Puppet Toads face threats to their survival, all have been discovered at forest edges, less than 1 km away from tea and/or coffee plantations, or mining pits. All of the *Sigalegalephrynus* species occur in isolated mountaintops where deforestation pressure is very high.

Deforestation rate in the provinces where *Sigalegalephrynus* toads are discovered is significantly high (Aceh 6.72%, Jambi 30.70%, West Sumatra 11.9% and South Sumatra

15.94%; Suprianta *et al.* 2017). These toads are under the direct and imminent threat of habitat destruction that warrant an immediate and strong conservation initiative. It is suggested to update the IUCN Red List status of the new species, given new information provided herein and IUCN Red List of Threatened Species category and criteria to at least Endangered, EN B1ab(iii) (IUCN Standards and Petitions Subcommittee 2017). Future research should focus on finding new species in areas with high presence probability and habitat suitability derived from our niche modeling analysis, as well as determining the distributional range of the identified species.

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AUTHOR CONTRIBUTIONS

GCS conducted analyses and wrote the manuscript. GCS, EW, PT, IS, & ENS conducted fieldwork and collected specimens. ENS, AH & NK obtained permission for conducting fieldwork in Indonesia. All authors have equal position in authorship, they also discussed, corrected and approved the final version of the manuscript.

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Table 1. GenBank Accession numbers of specimens used in molecular analysis.

Species	Location	Field Number	Voucher Number	GenBank Accession No. (16S)	Source
INGROUP					
Ansonia hanitschi	Malaysia; Borneo		VUB 0615	FJ882794	Van Bocxlaer *et al.* 2009
Ansonia leptopus	Malaysia; Borneo		VUB 0632	FJ882795	Van Bocxlaer *et al.* 2009
Ansonia sp.	Indonesia; Sumatra	ENS 19207	UTA A-65475	MH560504	This study
Ansonia spinulifer	Malaysia; Borneo		VUB 0647	FJ882798	Van Bocxlaer *et al.* 2009
Duttaphrynus melanostictus	Indonesia; Java	ENS 13607	UTA A-65510	MH560505	This study
Duttaphrynus melanostictus	Indonesia; Java	ENS 15036	UTA A-63417	MH560506	This study
Duttaphrynus melanostictus	Indonesia, Lampung	ENS 13762	UTA A-65511	MH560507	This study
Ingerophrynus biporcatus	Indonesia; Sumatra	ENS 7529	UTA A-53730	KX192090	Smart *et al.* 2017
Ingerophrynus divergens	Indonesia; Sumatra	ENS 18497	UTA A-65486	MH560508	This study
Leptophryne borbonica	Indonesia; Sumatra	ENS 14099	UTA A-62486	KX192095	Smart *et al.* 2017
Leptophryne cruentata	Indonesia; Java	ENS 15955	UTA A-62523	MH560509	This study
Pelophryne misera	Malaysia; Borneo		VUB 0641	FJ882800	Van Bocxlaer *et al.* 2009
Pelophryne signata	Malaysia; Borneo		VUB 0583	FJ882801	Van Bocxlaer *et al.* 2009

Pelophryne sp.	Indonesia; Sumatra	ENS 16092	UTA A-65485	MH560510	This study
Phrynoidis asper	Indonesia; Sumatra	ENS 15172	UTA A 63413	MH560511	This study
Phrynoidis asper	Indonesia; Java	ENS 16138	UTA A 63410	MH560512	This study
Phrynoidis juxtasper	Malaysia; Borneo		VUB 0649	FJ882805	Van Bocxlaer *et al.* 2009
Pseudobufo subasper	Indonesia; Sumatra	ENS 17047	UTA A-63763	KX192096	Smart *et al.* 2017
Pseudobufo subasper	Indonesia; Sumatra	ENS 17052	UTA A-63764	KX192093	Smart *et al.* 2017
Rentapia hosii	Malaysia; Borneo		BORNEENSIS 22088	AB331717	Matsui *et al.* 2007
Sabahphrynus maculatus	Malaysia; Borneo		BORNEENSIS 08425	AB331718	Matsui *et al.* 2007
Sigalegalephrynus burnitelongensis	Indonesia; Sumatra	ENS 18883	UTA A-65492	MH560517	This study
Sigalegalephrynus burnitelongensis	Indonesia; Sumatra	ENS 18884	MZB.Amph.30413	MH560518	This study
Sigalegalephrynus gayoluesensis	Indonesia; Sumatra	ENS 19525	UTA A-65490	MH560515	This study
Sigalegalephrynus gayoluesensis	Indonesia; Sumatra	ENS 19527	MZB.Amph.30411	MH560516	This study
Sigalegalephrynus harveyi	Indonesia; Sumatra	ENS 18377	MZB.Amph.30412	MH560513	This study
Sigalegalephrynus harveyi	Indonesia; Sumatra	ENS 18406	UTA A-65474	MH560514	This study
Sigalegalephrynus mandailinguensis	Indonesia; Sumatra	ENS 16936	UTA A-63562	KX192092	Smart *et al.* 2017
Sigalegalephrynus mandailinguensis	Indonesia; Sumatra	ENS 15697	MZB.Amph.25736	KX192094	Smart *et al.* 2017
Sigalegalephrynus minangkabauensis	Indonesia; Sumatra	ENS 16028	MZB.Amph.25738	KX192091	Smart *et al.* 2017
OUTGROUP					

Atelopus flavescens	French Guiana, S Kaw		BPN 726 (UTA)	DQ283259	Frost *et al.* 2006
Dryophytes arenicolor	USA; Mississippi	DCC 0343	TNHC 61118 (VUB 1052)	FJ882776	Van Bocxlaer *et al.* 2009

Table 2. Uncorrected P-distances between sequences based on 608 bp of 16S rRNA gene (percentages of base differences per site) (shaded regions represent intra-Group divergence and bold cells represent divergence between the southern and northern Groups).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	*Ansonia hanitschi (*VUB 0615)																															
2	*Ansonia leptopus* (VUB 0632)	9.2																														
3	*Ansonia sp.* (UTA A-65475)	8.5	2.8																													
4	*Ansonia spinulifer* (VUB 0647)	9.5	10.0	11.0																												
5	*Duttaphrynus melanostictus* (UTA A-65510)	10.0	10.6	11.1	12.6																											
6	*Duttaphrynus melanostictus* (UTA A-63417)	10.0	10.6	11.1	12.6	0.0																										
7	*Duttaphrynus melanostictus* (UTA A-65511)	10.0	10.6	11.1	12.6	0.0	0.0																									
8	*Ingerophrynus biporcatus* (UTA A-53730)	9.8	11.2	11.5	11.8	10.3	10.3	10.3																								
9	*Ingerophrynus divergens* (UTA A-65486)	12.6	12.8	12.4	13.7	10.5	10.5	10.5	8.2																							
10	*Leptophryne borbonica* (UTA A-62486)	12.3	13.3	12.1	13.0	11.8	11.8	11.8	11.5	13.4																						
11	*Leptophryne cruentata* (UTA A-62523)	14.4	16.0	15.8	15.2	13.4	13.4	13.4	13.6	13.9	12.2																					
12	*Pelophryne misera* (VUB 0641)	10.5	11.8	11.6	13.3	9.4	9.4	9.4	11.5	13.8	13.2	16.1																				
13	*Pelophryne signata* (VUB 0583)	10.2	10.5	10.5	12.0	10.7	10.7	10.7	11.1	14.0	10.2	15.7	6.5																			
14	*Pelophryne sp.* (UTA A-65485)	10.7	12.3	12.1	12.5	12.2	12.2	12.2	11.2	14.1	11.5	15.8	7.0	4.7																		
15	*Phrynoidis asper* (UTA A 63413)	12.2	10.6	11.1	11.3	9.0	9.0	9.0	10.8	11.6	11.7	12.5	12.0	13.2	13.3																	
16	*Phrynoidis asper* (UTA A 63410)	11.7	10.8	11.4	11.5	9.5	9.5	9.5	11.2	11.8	12.5	13.3	12.7	14.2	14.1	1.5																

17	*Phrynoidis juxtasper* (VUB 0649)	8.5	9.0	9.1	11.0	8.0	8.0	8.0	9.9	10.0	10.9	11.8	11.4	11.6	11.7	5.8	5.1															
18	*Pseudobufo subasper* (UTA A-63763)	10.7	11.5	11.7	13.0	9.5	9.5	9.5	10.2	11.5	10.7	14.5	13.8	13.7	14.1	9.7	9.4	9.9														
19	*Pseudobufo subasper* (UTA A-63764)	10.7	11.5	11.7	13.0	9.5	9.5	9.5	10.2	11.5	10.7	14.5	13.8	13.7	14.1	9.7	9.4	9.9	0.0													
20	*Pedostibes hosii* (BORNEENSIS 22088)	8.0	11.3	10.8	11.4	9.0	9.0	9.0	10.7	13.3	10.2	13.0	10.2	11.6	12.4	10.9	10.9	9.9	10.2	10.2												
21	*Sabahphrynus maculatus* (BORNEENSIS 08425)	9.8	12.1	10.9	11.3	8.8	8.8	8.8	9.2	9.1	10.5	12.4	11.0	11.4	11.0	11.3	11.9	9.3	9.2	9.2	9.5											
22	*Sigalegalephrynus burnitelongensis* (UTA A-65492)	12.8	14.2	15.1	13.3	11.6	11.6	11.6	13.1	12.7	12.7	14.3	12.2	12.2	12.8	11.2	11.7	12.0	12.3	12.3	11.4	13.1										
23	*Sigalegalephrynus burnitelongensis* (MZB.Amph.30413)	12.8	14.2	15.1	13.3	11.6	11.6	11.6	13.1	12.7	12.7	14.3	12.2	12.2	12.8	11.2	11.7	12.0	12.3	12.3	11.4	13.1	0.0									
24	*Sigalegalephrynus gayoluesensis* (UTA A-65490)	12.6	12.9	14.0	13.6	12.3	12.3	12.3	12.6	13.8	14.0	15.1	12.6	14.1	14.2	10.7	11.3	11.3	11.8	11.8	12.0	13.1	5.1	5.1								
25	*Sigalegalephrynus gayoluesensis* (MZB.Amph.30411)	12.6	12.9	14.0	13.6	12.3	12.3	12.3	12.6	13.8	14.0	15.1	12.6	14.1	14.2	10.7	11.3	11.3	11.8	11.8	12.0	13.1	5.1	5.1	0.0							
26	*Sigalegalephrynus harveyi* (MZB.Amph.30412)	9.7	11.0	9.8	11.4	8.7	8.7	8.7	10.0	11.3	9.2	11.7	10.7	12.1	12.2	7.8	8.0	8.2	9.2	9.2	7.5	8.8	9.2	9.2	8.2	8.2						
27	*Sigalegalephrynus harveyi* (UTA A-65474)	9.7	11.0	9.8	11.4	8.7	8.7	8.7	10.0	11.3	9.2	11.7	10.7	12.1	12.2	7.8	8.0	8.2	9.2	9.2	7.5	8.8	9.2	9.2	8.2	8.2	0.0					
28	*Sigalegalephrynus mandailinguensis* (MZB.Amph.25736)	11.4	10.7	11.0	11.6	9.0	9.0	9.0	10.2	10.8	9.3	12.2	11.1	11.3	11.7	7.3	8.2	7.0	10.1	10.1	9.6	10.4	9.3	9.3	8.9	8.9	4.0	4.0				
29	*Sigalegalephrynus mandailinguensis* (UTA A-63562)	11.4	10.7	11.0	11.6	9.0	9.0	9.0	10.2	10.8	9.3	12.2	11.1	11.3	11.7	7.3	8.2	7.0	10.1	10.1	9.6	10.4	9.3	9.3	8.9	8.9	4.0	4.0	0.0			
30	*Sigalegalephrynus minangkabauensis* (MZB.Amph.25738)	12.4	12.8	11.8	13.2	10.5	10.5	10.5	12.9	13.3	10.1	13.3	11.9	12.6	12.9	8.5	9.0	9.2	10.7	10.7	9.6	11.0	10.4	10.4	10.4	10.4	4.7	4.7	6.0	6.0		
31	*Atelopus flavescens* (BPN 726)	16.0	13.8	13.0	15.1	15.4	15.4	15.4	13.5	16.1	15.3	17.3	17.9	16.7	16.7	13.0	13.8	12.5	17.1	17.1	16.2	13.8	17.4	17.4	18.0	18.0	14.3	14.3	13.5	13.5	16.8	
32	*Dryophytes arenicolor* (TNHC 61118 (VUB 1052)	14.5	17.1	17.2	15.6	12.6	12.6	12.6	12.6	15.6	16.3	17.3	16.7	16.0	15.8	12.9	14.3	14.1	12.9	12.9	14.8	14.3	15.9	15.9	14.9	14.9	13.7	13.7	14.5	14.5	15.6	15.5

Table 3. Area of occupancy (AOO) and extent of occurrence (EOO) output with suggested IUCN Red List Status from GeoCAT analysis.

Species	IUCN default value				Default IUCN value*2			
	EOO (km^2)	AOO (km^2)	EOO Status	AOO Status	EOO (km^2)	AOO (km^2)	EOO Status	AOO Status
S. harveyi	0	4	CR	CR	0	16	CR	EN
S. minangkabauensis	0	4	CR	CR	0	4	CR	EN
S. mandailinguensis	0.067	8	CR	CR	0.067	32	CR	EN
S. gayoluesensis	0.017	8	CR	CR	0.017	16	CR	EN
S. burnitelongensis	0	8	CR	CR	0	32	CR	EN

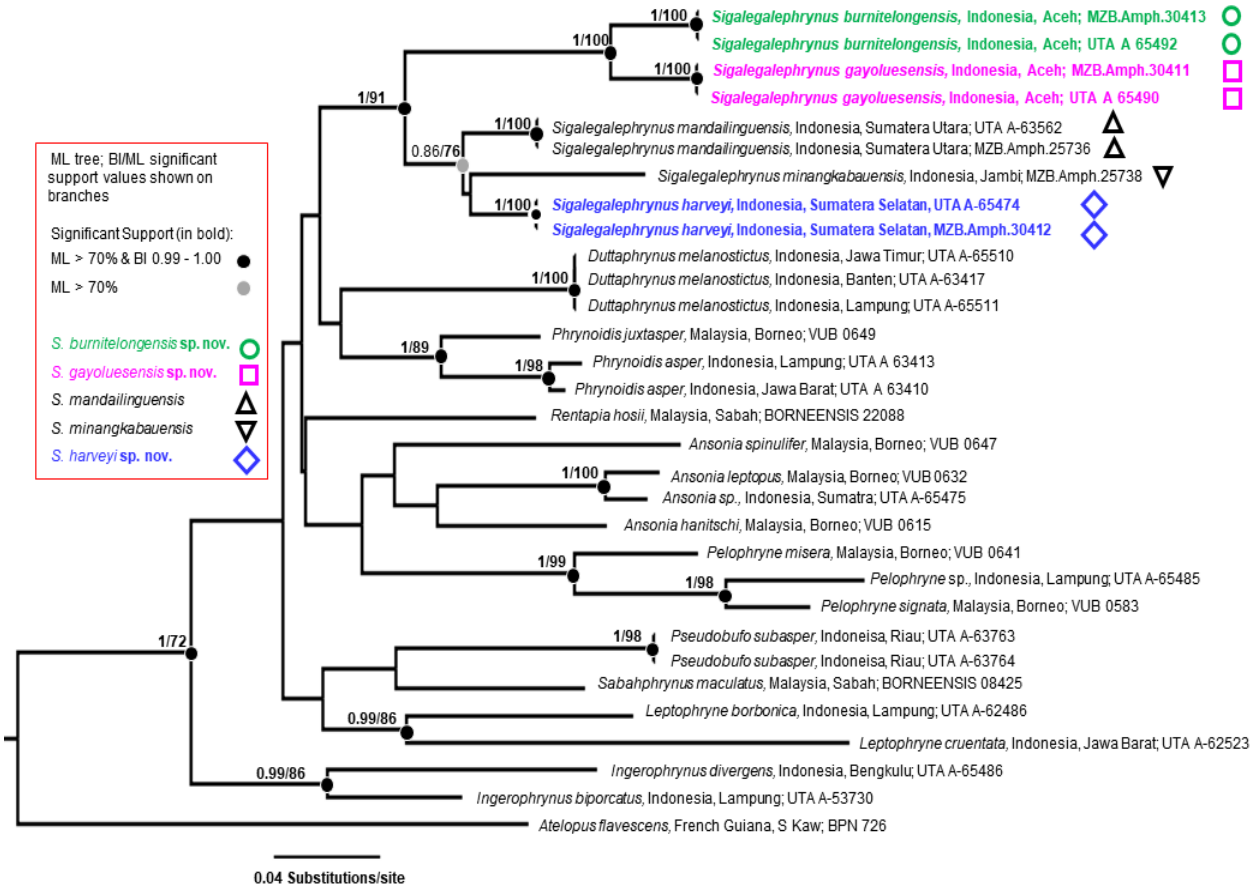

Figure 1. Estimated phylogeny of *Sigalegalephrynus* based on 16S mitochondrial rRNA, depicted as a maximum-likelihood tree with bufonid outgroups. The non-bufonid outgroup *Dryophytes arenicolor* is not shown.

Figure 2. Lateral, dorsal, and ventral views of specimens of *Sigalegalephrynus* in life. Holotypes of *S. burnitelongensis* (A–C, MZB.Amph.30413, SVL 22.18 mm), S. *gayoluesensis* (D–F, MZB.Amph.30411, SVL 26.49 mm), *S. mandailinguensis* (G–I, MZB.Amph.25736, SVL 38.01 mm), *S. minangkabauensis* (J–L, MZB.Amph.25738, SVL 19.32 mm), and *S. harveyi* (M–O, MZB.Amph.30412, SVL 26.36 mm).

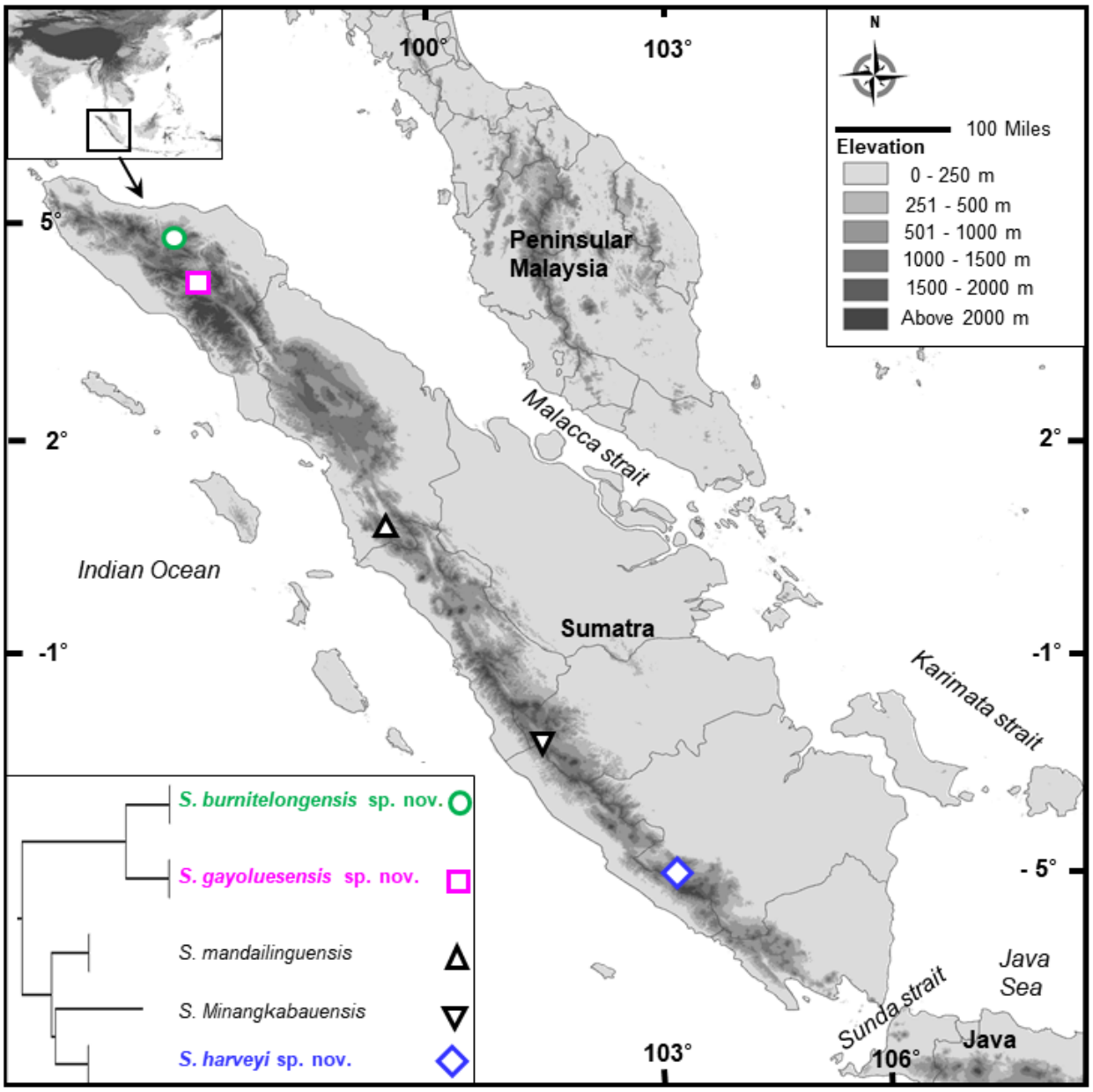

Figure 3. Map of Sumatra showing the known distribution of *Sigalegalephrynus* species were found.

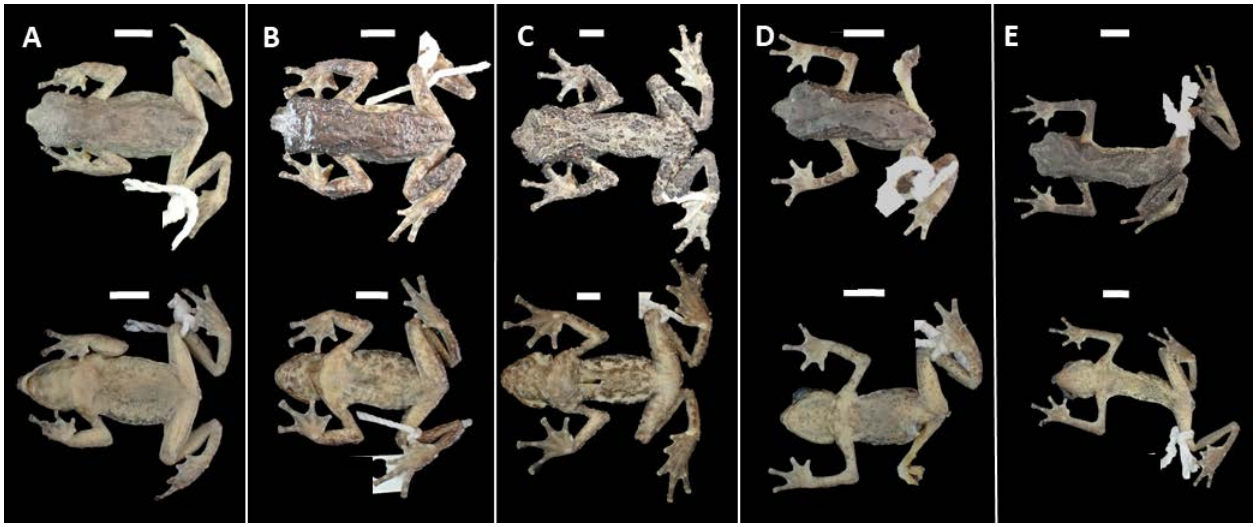

Figure 4. Dorsal (top) and ventral (bottom) aspects of *Sigalegalephrynus* specimens in alcohol (Scale bar = 5 mm). Holotypes of *Sigalegalephrynus burnitelongensis* (A, MZB.Amph.30413), *S. gayoluesensis* (B, MZB.Amph.30411), *S. mandailinguensis* (C, MZB.Amph.25736), *S. minangkabauensis* (D, MZB.Amph.25738), and *S. harveyi* (E, MZB.Amph.30412).

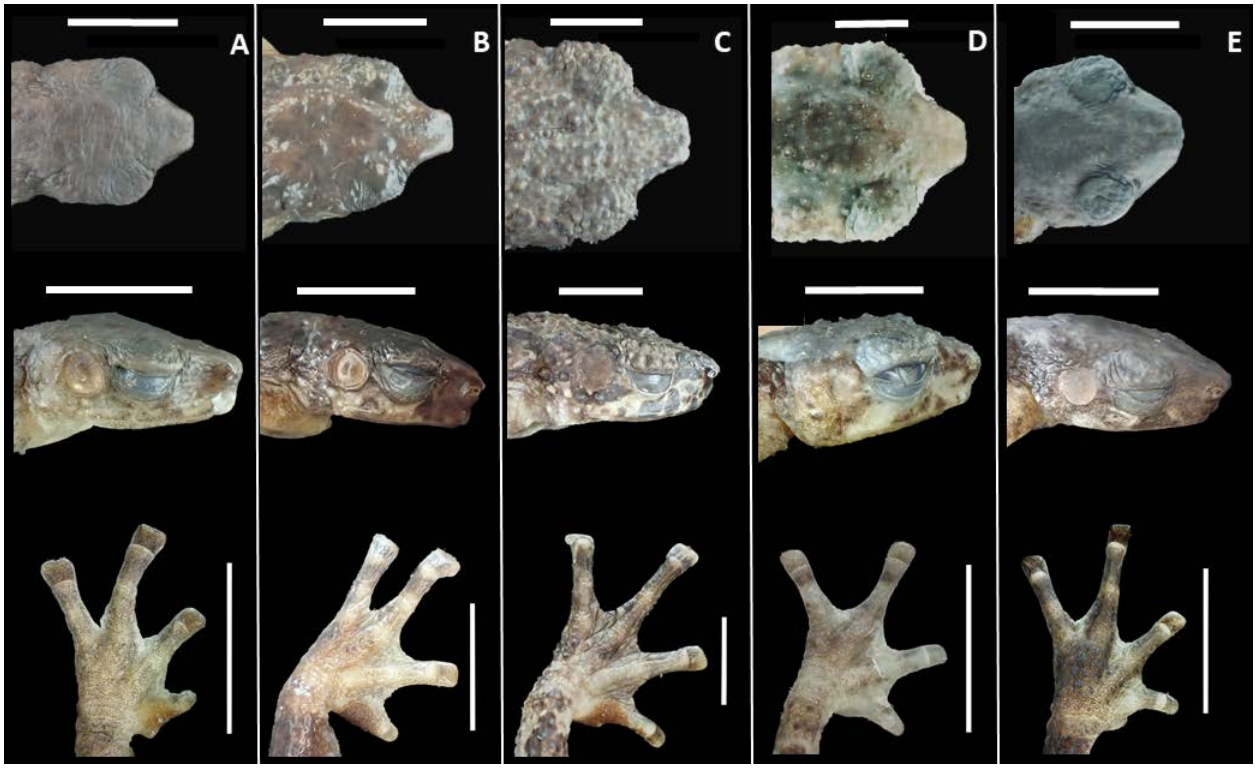

Figure 5. Dorsal (top) and lateral (middle) profiles of *Sigalegalephrynus* specimens in alcohol (Scale bar = 5 mm). Head, and upper surface of hand (bottom) of holotypes of *Sigalegalephrynus burnitelongensis* (A, MZB.Amph.30413), *S. gayoluesensis* (B; MZB. (B; MZB.Amph.30411), *S. mandailinguensis* (C, MZB.Amph.25736), *S. minangkabauensis* (D, MZB.Amph.25738), and *S. harveyi* (E, MZB.Amph.30412).

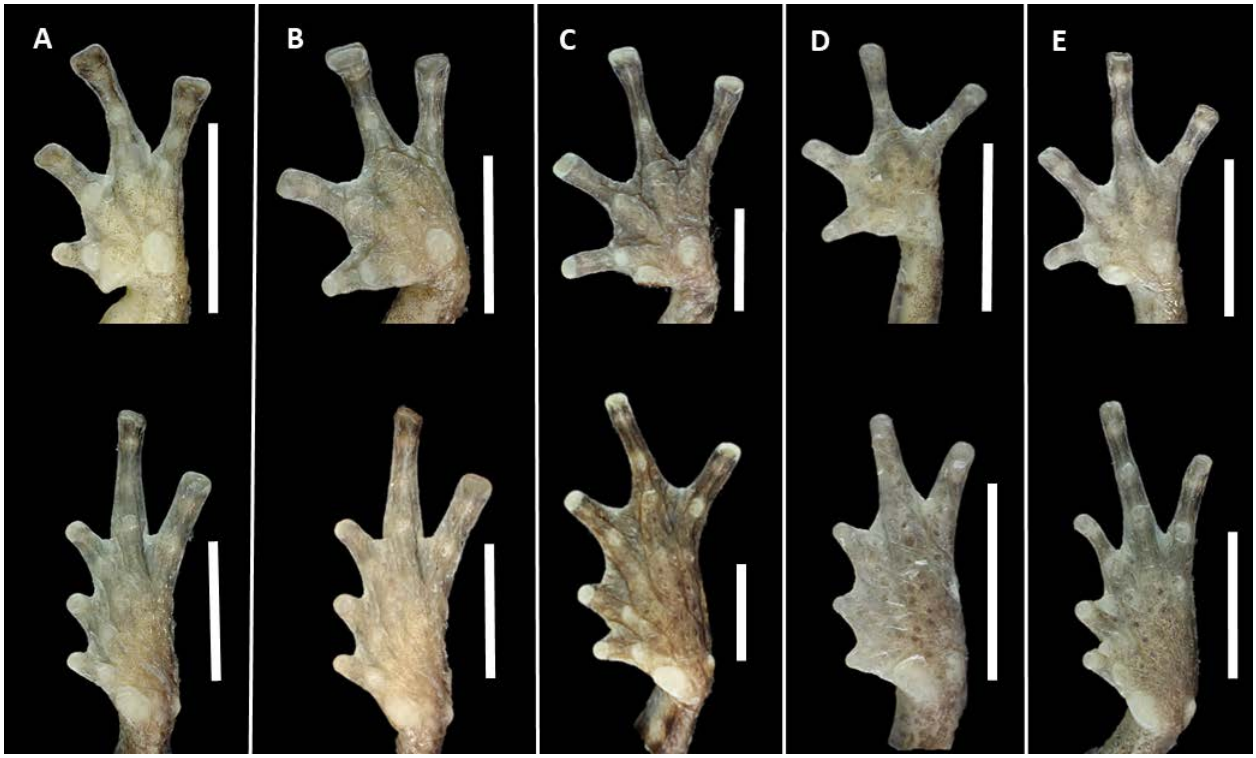

Figure 6. Palmar and plantar surfaces of *Sigalegalephrynus* specimens in alcohol (Scale bar = 5 mm). Holotypes of *Sigalegalephrynus burnitelongensis* (A, MZB.Amph.30413*), S. gayoluesensis* (B, MZB.Amph.30411), *S. mandailinguensis* (C, MZB.Amph.25736), *S. minangkabauensis* (D, MZB.Amph.25738), and *S. harveyi* (E, MZB.Amph.30412).

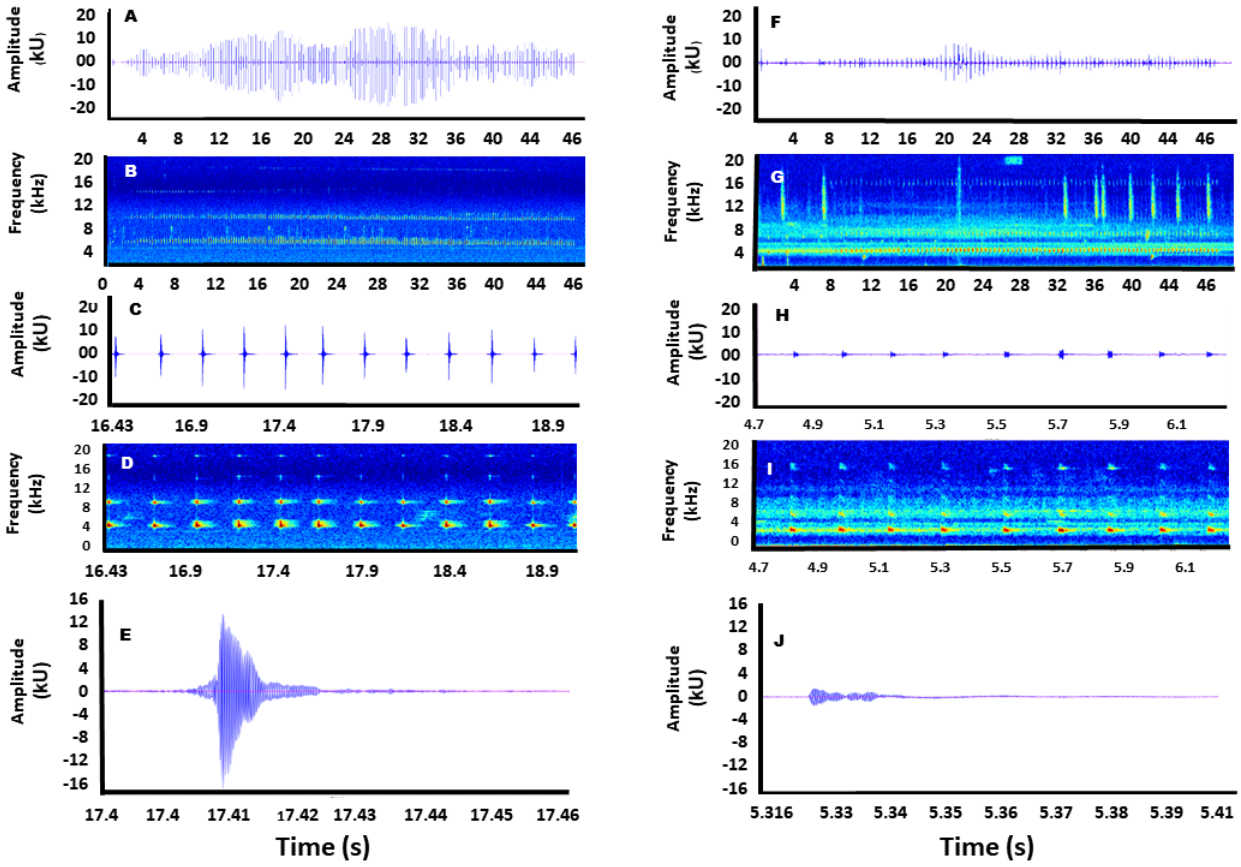

Figure 7. Spectral graphs of known calls for species of *Sigalegalephrynus*, *S. gayoluesensis* (MZB.Amph.30411) and *S. mandailinguensis* (MZB.Amph.25736). Oscillograms (A and C, *S. gayoluesensis*; F and H, *S. mandailinguensis*); spectrograms (B and D, *S. gayoluesensis*; G and I, *S. mandailinguensis*); oscillograms of a single pulse within call (E, *S. gayoluesensis*; J, *S. mandailinguensis*).

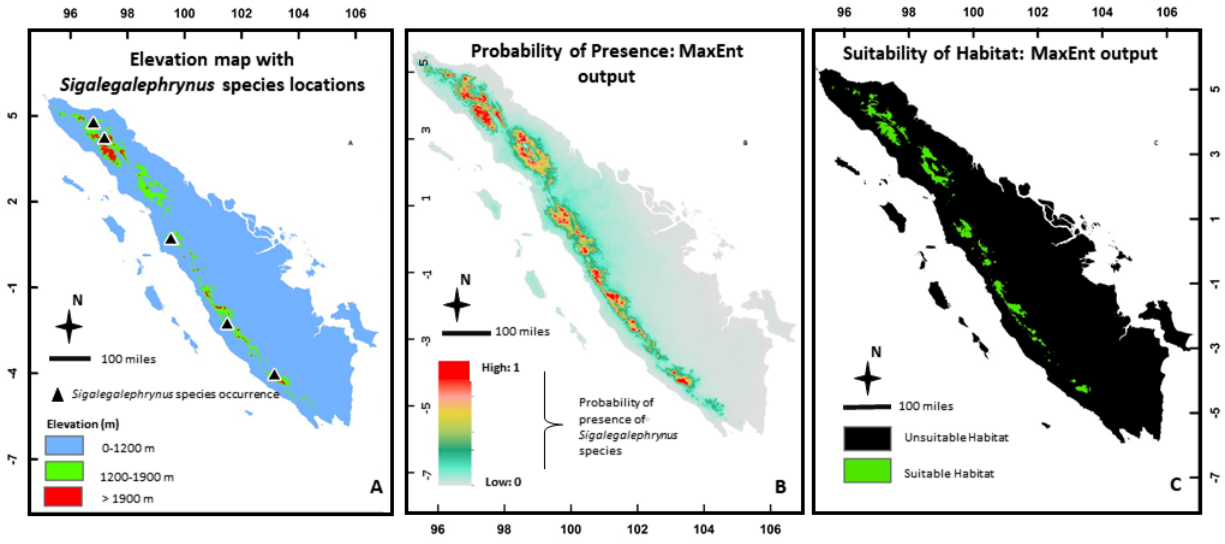

Figure 8. A) Elevational distribution of *Sigalgalephrynus* species in Sumatra. B) Map showing probability of presence of *Sigalegalephrynus* species. C) Map showing suitable habitats of *Sigalegalephrynus* species (according to 10 percentile rule in MaxEnt).

APPENDIX

Appendix I. Additional Specimens Examined.

Sigalegalephrynus mandailinguensis (4).—INDONESIA: Sumatera Utara: Kabupaten Mandailing Natal: Gunung Sorikmarapi (W side), 1383 m. a.s.l., 0.701648ºN, 99.552628ºE, MZB.Amph.25736—Holotype (male); trail between the Tano Bato to Sapo Tinjak road and Lake Saba Begu, Batang Gadis National Park, 1297 m. a.s.l., 0.708668ºN, 99.519538ºE, MZB.Amph.25737—Paratype (male); 1299 m. a.s.l., 0.708668ºN, 99.519538ºE, UTA A63562—Paratype (male), UTA A63561—Paratype (male).

Sigalegalephrynus minangkabauensis (1).—INDONESIA: Jambi: Kabupaten Kerinci: Gunung Kunyit, 1428 m. a.s.l., 2.260138ºS, 101.495128ºE, MZB.Amph. 25738—Holotype.

Appendix II. Published article on the genus *Sigalegalephrynus*

Herpetologica, 73(1), 2017, 63–75

A New Genus and Two New Species of Arboreal Toads from the Highlands of Sumatra with a Phylogeny of Sundaland Toad Genera

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Abstract: We describe a new genus and two new species of toads from the Sumatran volcanoes Gunung Sorikmarapi and G. Kunyit, in the provinces of Sumatera Utara and Jambi, respectively. The new taxa can be distinguished from other genera, and each other, based on genetic differentiation, morphology, and advertisement call structure. We employ both nuclear and mitochondrial data to provide a phylogenetic hypothesis of relationships for the bufonid genera of the Sunda Shelf. While broadly corroborating previous studies, our results also shed light on the phylogenetic position of *Pseudobufo*. The new genus, *Duttaphrynus*, and *Pseudobufo* are basal to other Sunda Shelf genera in our phylogenies.

Key words: Barisan Range; Bufonidae; Molecular phylogenetics; Sunda Shelf; Taxonomy

Between June 2013 and February 2014, we carried out an inventory of the highland herpetofauna of Sumatra. One trip, in January 2014, revealed a very peculiar species of unidentified toads from the slopes of Gunung Sorikmarapi, a densely vegetated stratovolcano situated in Batang Gadis National Park, in the Mandailing Natal Kabupaten of the Sumatera Utara Province. We found the first specimen inside a small subterranean hollow, crouching motionless on a rock wall, ~2 m below the ground. Further searching inside caves in the area did not reveal more individuals. Nonetheless, we found three more specimens at nearby localities the following day. While comparing these specimens to others collected from the previous year, we came across another individual that was collected at Gunung Kunyit of the Barisan Range in Jambi Province, Sumatra in June 2013, which exhibited close morphological affinities to the peculiar specimens from Batang Gadis National Park.

Here we appraise these specimens within the framework of a lineage-based, Unified Species Concept (de Queiroz 2005). Using morphological diagnosability, phylogenetic relationships, genetic divergence, and patterns of advertisement calls for assessing the evolutionary independence of this lineage, we provide several lines of evidence to validate the recognition of this lineage as a new genus and its constituent taxa as two new species. We also take this opportunity to clarify the genus-level phylogeny of the toads found on the Sunda Shelf.

Materials and Methods

Morphology

Specimens used in this work were collected during fieldwork in Sumatra and Java between May 2013 and August 2015. We photographed specimens when alive and then again after they were fixed according to Institutional Animal Care and Use Committee protocols. We took dorsal, ventral, and lateral photographs of the specimens post mortem and with a scale for size reference. We preserved specimens in a 1:9 dilution of stock formalin in water until they could be transferred to 70% ethanol. Finally, we deposited specimens at the Museum Zoologicum Bogoriense (MZB), Cibinong, Indonesia, and the Amphibian and Reptile Diversity Research Center of the University of Texas at Arlington (UTA), Texas, USA.

We used morphological terminology primarily based on Matsui (1984), while also using terminology of Duellman (2001) and Kok and Kalamandeen (2008). With a digital caliper (≥3 mm) or ocular micrometer (<3 mm) to the nearest 0.1 mm, we measured: snout–vent length (SVL); head length; head width (width of the head measured at the angles of the jaw, excluding warts at the jaw); snout length (from the anterior ocular angle to the tip of the snout); intercanthal distance (distance between anterior edges of canthus); internarial distance (distance between proximal ends of nares); interorbital distance (the minimal distance between the upper eyelids); eye length (distance between the anterior and posterior adjunction of upper and lower eyelids); eye–nares length (distance from the proximal junction of upper and lower eyelids and proximal end of nares); nares–snout length (distance between the proximal end of nares to the tip of the snout); tympanum width (horizontal); forearm length (from tip of the elbow to the proximal margin of outer metatarsal tubercle); hand length (from the distal margin of the metatarsal tubercle to the tip of the third finger); femur length, tibia length (the greatest length of tibia by positioning the hind limb in a Z pattern); tarsus length (distance of tibio-tarsal joint to the proximal end of outer metatarsal tubercle); foot length (from the distal margin of the outer metatarsal tubercle to the tip of Toe IV); and finger pad width (greatest width of finger pad). We used the webbing formulae of Myers and Duellman (1982). We used digital images to describe color in life; these images are

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TABLE 1.—Specimens used in a molecular analysis of bufonid anurans from the Sunda Shelf, Malaysia. If museum numbers are unavailable, we provide the field number (SDB) or the tissue collection number (VUB). New sequences are indicated in bold.

Species	Locality	Museum number	Genbank accession number			
			12S	16S	NCX1	CXCR4
Outgroup						
Dryophytes (Hyla) arenicolor	USA; Arizona	TNHC 61118 (VUB 1052)	FJ882776	FJ882776	EF107241	AY364190
Anaxyrus americanus	USA; Mississippi	CAS 207258	FJ882827	FJ882827	FJ882676	FJ882730
Ingroup						
Ansonia leptopus	Malaysia; Borneo	VUB 0632	FJ882795	FJ882795	—	—
Ansonia penangensis	Malaysia; Penang	KUHE UNL1	AB435262	AB435262	—	—
Duttaphrynus melanostictus	Indonesia; Java	UTA 53737	**KX192078**	**KX192086**	**KX19211**	—
Ingerophrynus biporcatus	Indonesia; Sumatra	UTA 53730	**KX192079**	**KX192090**	**KX19212**	—
Ingerophrynus divergens	Malaysia; Borneo	VUB 0602	FJ882802	FJ882802	FJ882648	FJ882701
Leptophryne cruentata	Indonesia; Java	UTA 62522	**KX192075**	**KX192087**	**KX19207**	**KX192108**
Leptophryne borbonica	Indonesia; Sumatra	UTA 62486	**KX192076**	**KX192095**	**KX19208**	—
Pedostibes tuberculosus	India; Western Ghats	SDB 4691	FJ882793	FJ882793	FJ882640	FJ882693
Pelophryne brevipes	Indonesia; Sumatra	UTA 63762	**KX192080**	**KX192088**	**KX19210**	—
Pelophryne signata	Malaysia; Borneo	VUB 0583	FJ882801	FJ882801	FJ882646	FJ882699
Phrynoidis juxtasper	Malaysia; Borneo	VUB 0649	FJ882805	FJ882805	FJ882656	FJ882710
Phrynoidis asper	Indonesia; Java	UTA 53719	**KX192077**	**KX192089**	**KX19209**	—
Pseudobufo subasper	Indonesia; Sumatra	UTA 63763	**KX192083**	**KX192096**	**KX19216**	—
Pseudobufo subasper	Indonesia; Sumatra	UTA 63764	**KX192084**	**KX192093**	**KX19217**	—
Rentapia hosii	Malaysia; Borneo	VUB 0661	FJ882804	FJ882804	EF107223	EF107449
Sabahphrynus maculatus	Malaysia; Sabah	BORNEENSIS 08425	AB331718	AB331718	—	—
Sigalegalephrynus mandailinguensis gen. nov., sp. nov.	Indonesia; Sumatra	UTA 63562	**KX192082**	**KX192092**	**KX19215**	**KX192110**
Sigalegalephrynus mandailinguensis gen. nov., sp. nov.	Indonesia; Sumatra	MZB 25736	**KX192081**	**KX192094**	**KX19214**	**KX192109**
Sigalegalephrynus minangkabauensis gen. nov., sp. nov.	Indonesia; Sumatra	MZB 25738	**KX192085**	**KX192091**	**KX19213**	—

deposited at the University of Texas at Arlington (UTA) digital image collection. We followed the museum acronyms available in Sabaj Perez (2014).

Molecular Phylogenetic Analyses

Taxon sampling and DNA sequencing.—We included 21 individual operational taxonomic units of 13 distinct toad genera in this study (Table 1). Apart from the three undescribed specimens, this data matrix also included sequences from type species of all genera of toads that occur in the Sunda Shelf, except for *Pelophryne* whose type species, *P. albotaeniata* (Barbour 1938), lacks sequence data. In our matrix, *Pelophryne* was thus represented by *P. brevipes* and *P. signata*. To minimize the effect of long-branch attraction, and to help stabilize the phylogeny (see Bergsten 2005), we also included one species of Sumatran toad from each genus alongside the types where possible. *Pedostibes hosii* was recently made the type species for the new genus *Rentapia* (Chan et al. 2016), but previously belonged to a genus that encompassed an Indian endemic (*Pedostibes tuberculosus*, type species of *Pedostibes*) and four Southeast Asian arboreal toads (Frost et al. 2006). We decided to incorporate *Pedostibes tuberculosus* in our study, to contextualize our phylogeny in the light of this recent taxonomic development. To minimize missing data, we chose not to include *R. rugosus* in our analysis because only the small ribosomal subunits 12S and 16S are currently available for this species on GenBank. Our sampling of Bufonidae thus incorporated species of the following genera: *Ansonia* (*A. penangensis* type species and *A. leptopus*); *Duttaphrynus* (*D. melanostictus* type species); *Ingerophrynus* (*I. biporcatus* type species and *I. divergens*); *Leptophryne* (*L. cruentata* type species and *L. borbonica*); *Pedostibes* (*P. tuberculosus* type species); *Rentapia* (*R. hosii* type species); *Pelophryne* (*P. signata* and *P. brevipes*); *Sabahphrynus* (*S. maculatus* type species); the monotypic genus *Pseudobufo* (*P. subasper* type species); and two undescribed specimens from Gunung Sorikmarapi and one undescribed specimen from Gunung Kunyit. We also included American Toads (*Anaxyrus americanus*) and Canyon Treefrogs (*Dryophytes* [*Hyla*] *arenicolor*) as outgroup taxa (Duellman et al. 2016). Here, we provide new sequences for 11 individuals; the remaining 10 were published by Van Bocxlaer et al. (2009) and obtained from GenBank.

Prior to preservation of specimens, we collected either muscle or skin tissue and stored it in 1.5 mL of cell lysis buffer solution (0.5 M Tris/0.25% EDTA/2.5% SDS, pH = 8.2) or in 95% ethanol. We isolated Genomic DNA using a Qiagen DNeasy kit (Qiagen, Valencia, CA, USA) or AMPure XP beads following the Agencourt protocol (Beckman Coulter Co., Fort Collins, CO, USA) after Rohland and Reich (2012). We sequenced 2 mitochondrial (12S and 16S) and 2 nuclear genes (NCX1 and CXCR4), slightly modifying the primers and thermocycle protocols provided by Van Bocxlaer et al. (2009). We used these loci because they have been screened by previous studies on bufonids and have been established as informative (Biju et al. 2009; Van Bocxlaer et al. 2009, 2010). We used the GoTaq® Green Master Mix, 2X (Promega Corporation, Madison, WI, USA) for all amplification reactions on a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). We checked all successful polymerase chain reaction products visually in agarose gels and purified them using AMPure XP beads following the Agencourt protocol. The Genomic Core Facility at the University of Texas at Arlington completed the sequencing reactions with an ABI PRISM 3100xl Genetic Analyzer (Applied Biosystems).

Sequence alignment and phylogeny inference.—We assembled and cleaned the raw gene-fragment sequences using Sequencher (v4.8; Gene Codes, Ann Arbor, MI, USA). Prior to alignment, we used the Gblocks Server (v0.91b; Castresana 2000; Talavera and Castresana 2007) to eliminate poorly aligned and hyper-variable regions present in the ribosomal subunits. We then obtained the alignment of sequences using Clustal W (Larkin et al. 2007) implemented in MEGA (v5.1; Tamura et al. 2011). We translated the sequences of the protein coding genes to amino acid sequences, to verify the absence of stop codons and proper alignment, and where necessary, we edited them by eye for accuracy. We did not detect any internal stop codons and we deposited the new sequences in GenBank (accession numbers in Table 1).

We partitioned the concatenated data set by gene and codon position and used Partition-Finder (v1.1.0; Lanfear et al. 2012) to determine the best partitioning schemes and models for each partitioned subset. Partition-Finder simultaneously determined the partition schemes based on Bayesian Information Criterion using the "greedy" search algorithm. The best partitioning scheme had seven partitions: 12S and 16S, GTR+I+Γ; CXCR4 codon 3, HKY+I; CXCR4 codon 1, HKY+G; CXCR4 codon 2, GTR; NCX1 codon 2, HKY+I; NCX1 codon 3, GTR+Γ; and NCX1 codon 1, HKY+I+ Γ.

To examine the phylogenetic position of the undescribed species in relation to Sundaland toads, we used maximum likelihood (ML) and Bayesian inference (BI) methods. We conducted ML analysis employing the rapid bootstrapping algorithm using the program RAxML v8.00 (Stamatakis 2014) on the CIPRES Science Gateway server (v3.2; Miller et al. 2010). Because the 25 discrete rate categories are said to better approximate invariant sites (Stamatakis 2006), we used the model GTR+G instead of GTR+I+G for the ML analysis. Nodal support for ML was provided by bootstrapping (BS; 1000 pseudoreplicates), with BS values $\geq$0.70 considered strong support (Hillis and Bull 1993).

We conducted Bayesian Markov chain Monte Carlo (MCMC) phylogenetic analyses on a partitioned alignment using MrBayes (v3.3; Ronquist and Huelsenbeck 2003). We initiated two simultaneous runs of four MCMC analyses, consisting of one cold and three incrementally heated chains, with random trees for 5×10^6 total generations (sampling every 500 generations). We set the burn-in to the default values of 25%, hence discarding the initial 2500 generations. To examine stationarity, we used trace plots and ESS values (>200) on TRACER v1.5 (Rambaut and Drummond 2009). We constructed a 50% majority-rule consensus tree with estimates of Bayesian support using the remaining sampled trees and posterior probabilities (PP), wherein PP values $\geq$0.95 were considered strong support (Alfaro et al. 2003; Huelsenbeck and Rannala 2004; Mulcahy et al. 2011). We used the graphical viewer Figtree (Rambaut 2007) to edit the resulting output of RAxML and MrBayes analyses.

Advertisement Call Analysis

Elijah Wostl recorded the vocalization of an uncaptured individual from near Lake Saba Begu on 26 January 2014. He recorded a single call at 2156 h using a Zoom H4n Handy Recorder at a sampling rate of 44.1 kHz. The ambient temperature at the time of the recording was 18.7°C. To remove background noise, we first filtered the recording using the free sound-editing software Audacity (v2.0.3; Audacity Team 2014).

We analyzed the temporal and spectral characteristics of the recorded vocalizations using the sound synthesis and analysis package Seewave (v1.7.3; Sueur et al. 2008) implemented in RStudio (v0.98.1062; R Core Team 2013). We measured four traditional call characters known to be important in communication, including dominant frequency, call duration, pulse duration, and pulse rate (Cocroft and Ryan 1995). We estimated dominant frequency in Seewave using a fast Fourier transformation (Hanning window length = 100 samples; 85% overlap between successive windows). We obtained two-dimensional spectrograms using the function spectro. We measured temporal properties using the function timer, with a 2% amplitude threshold for signal detection for pulse duration and pulse rate.

RESULTS

Phylogenetic Analyses

Our final data matrix consisted of 1537 base pairs and was 94% complete, with only two species lacking both nuclear loci (*Ansonia penangensis* and *Sabahphrynus maculatus*) and one individual of the undescribed species from Gunung Sorikmarapi for which we were unable to amplify the CXCR4 exon. Our ML analyses produced a single tree (lnL = –6110.25; Fig. 1) that is almost identical to our Bayesian consensus phylogram. Most basal nodes are poorly supported; hence, the relationships among the Sundaland toad genera remain essentially unresolved. Each genus is recovered as monophyletic with high support. The group containing the undescribed individuals from Sumatra is nested within the Sundaland toads and forms a well-supported clade (BS = 100; PP = 1) with the two specimens from Gunung Sorikmarapi being sister to a more divergent specimen from Gunung Kunyit (uncorrected "*p*" distance = 4.2%; Table 2; BS = 100; PP = 1). We recovered a strongly supported sister relationship between *Pedostibes tuberculosus* and *Duttaphrynus melanostictus* (BS = 80; PP = 98), and together these two genera form a sister relationship with all Sundaland bufonids (BS = 14; PP = 0.96). We also recovered a moderately strong sister relationship between *Rentapia hosii* and frogs of the genus *Phrynoidis* (BS = 52; PP = 96), again in concordance with previous molecular phylogenies (Matsui et al. 2007; Van Bocxlaer et al. 2009, 2010; Pyron and Wiens 2011; Chan et al. 2016). Our analyses recovered the genus *Ansonia* as sister to a clade containing *Pelophryne* and *Ingerophrynus* with modest support in both analyses (BS = 25; PP = 0.77). *Ansonia* and *Pelophryne* have been recovered as sister taxa before (Van Bocxlaer et al. 2009; Pyron and Wiens 2011) but to the exclusion of *Ingerophrynus*. Our analyses did not confirm the sister relationship between *Ingerophrynus* and *Sabahphrynus* (contra Van Bocxlaer et al. 2009; Pyron and Wiens 2011), but instead indicated weak support for the latter as sister to *Leptophryne* (BS = 14; PP = 0.47). The monotypic genus *Pseudobufo* aligned with *D. melanostictus* and *P. tuberculosus* in our ML tree (BS = 20); whereas in our BI tree, it was recovered as sister to all Sundaland toads (PP = 0.44). Despite poor support, both analyses assign a basal position to *Pseudobufo* relative to all other Sundaland bufonid genera.

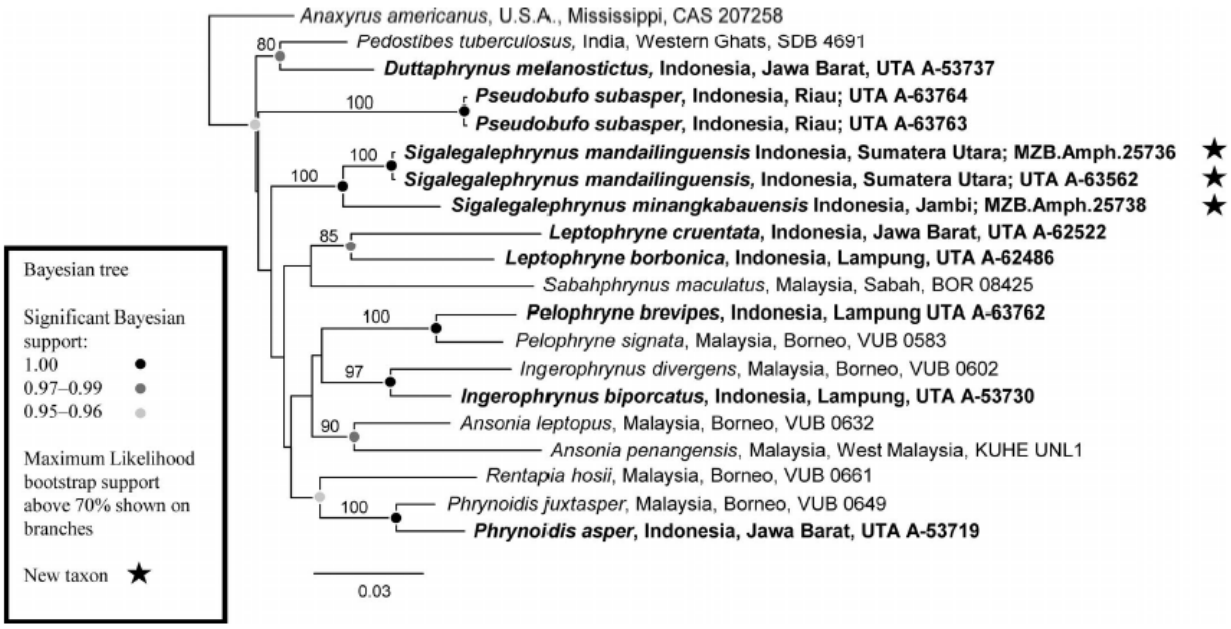

FIG. 1.—Phylogeny of Sundaland toads, based on 1537 base pairs of a combined mitochondrial and nuclear data set, depicted as a maximum-likelihood consensus tree with *Anaxyrus americanus* as a proximate outgroup (the nonbufonid outgroup *Dryophytes* [*Hyla*] *arenicolor* has been trimmed out).

DESCRIPTION OF NEW GENUS

The results of our phylogenetic analyses, as well as the call pattern data, indicate that the two undescribed species of toads form a distinct lineage among Southeast Asian bufonids. Therefore, we propose to establish a new genus for these two species.

Sigalegalephrynus gen. nov.

Type species.—*Sigalegalephrynus mandailinguensis* by present designation.

Diagnosis and comparisons.—The genus can be diagnosed based on the following ecological attributes and morphology: medium-sized (<40 mm SVL) member of Bufonidae, with gangly limbs, and arboreal and troglodytic habits. The morphologically comparable genus *Ansonia* (>40 mm SVL), commonly called Stream toads, is typically found on low vegetation near watercourses. The generally diminutive members of *Pelophryne* (<40 mm SVL) and *Leptophryne* (>40 mm SVL) occur on shrubs. The only other genus of toads that has true arboreal habits is *Rentapia*, whose members typically tend to be stockier (>70 mm SVL) than *Sigalegalephrynus*. The genus *Phrynoidis* is represented by two large (>70 mm SVL) semiaquatic toads, usually found on rocks along streams and rivers; the members of the *Duttaphrynus* (>40 mm SVL) and *Ingerophrynus* (>40 mm SVL) display terrestrial or somewhat riparian habits. The monotypic genus *Pseudobufo* is represented by a large (>75 mm SVL) and aquatic species with completely webbed feet that inhabits the peat swamps of the Malay Peninsula, Borneo, and Eastern Sumatra.

Like all other Sundaland toad genera (characters in parentheses), the new genus possesses a visible or slightly visible tympanum. *Sigalegalephrynus* most closely resembles *Ansonia;* however, the former lacks mandibular spines (mandibular spines present) and possesses combined femur and tibia lengths smaller than its SVL (SVL < femur and tibia lengths). Unlike *Pelophryne*, in *Sigalegalephrynus* Finger I projects beyond the webbing by two phalanges (reduced Finger I, with one or no phalanges projecting beyond webbing), and males possess nuptial excrescences with well-keratinized spicules (poorly spiculated with only slight keratinization or not keratinized at all). Unlike *Rentapia*, *Sigalegalephrynus* lacks paratoid glands (paratoids prominent). The new genus can be told apart from *Ingerophrynus* by the lack of well-defined parallel crests between the eyes (parallel crests prominent). Unlike

TABLE 2.—Pairwise genetic distances (uncorrected *p*) observed in the sequence of the mitochondrial 16S ribosomal subunit gene between two new species of puppet toads, and between arboreal toad genera of Sundaland.

Pairwise comparison	% difference
S. mandailinguensis to *S. minangkabauensis*	4.2
Sigalegalephrynus to *Pelophryne*	9–9.55
Sigalegalephrynus to *Leptophryne*	7.55–8.75
Sigalegalephrynus to *Ansonia*	8.6
Sigalegalephrynus to *Ingerophrynus*	6.55–8.25
Sigalegalephrynus to *Rentapia*	8.6
Sigalegalephrynus to *Pedostibes*	6.1
Sigalegalephrynus to *Phrynoidis*	6.75–6.95
Sigalegalephrynus to *Sabahphrynus*	8.2
Sigalegalephrynus to *Duttaphrynus*	6.85
Sigalegalephrynus to *Pseudobufo*	8.0
Pelophryne to *Leptophryne*	8–10.85
Pelophryne to *Ansonia*	10.1
Pelophryne to *Rentapia*	9.9
Pelophryne to *Sabahphrynus*	10.5
Ansonia to *Rentapia*	7.0
Ansonia to *Sabahphrynus*	9.4
Sabahphrynus to *Rentapia*	9.4

Leptophryne, *Sigalegalephrynus* lacks enlarged tubercles at the base of each toe, between at the articulation of the first phalanx and metacarpus (large tubercles present). Additionally, males of *L. cruentata* are unique among Southeast Asian toads in having nuptial excrescences that are white and swollen on the first and second fingers; males of *Sigalegalephrynus* (and all other genera) have nuptial excrescences with no white and swollen tissue. Unlike *Phrynoidis*, *Sigalegalephrynus* has slender limbs (limbs robust) and toes that are less than half webbed (toes fully webbed, with the exception of the fourth). Unlike *Pseudobufo*—the only Sundaland toad with fully webbed toes—*Sigalegalephrynus* has toes that are less than half webbed.

Males of *Sigalegalephrynus* can be distinguished from all other toads in the region by the presence of an elongate inner metacarpal-thenar tubercle, which is as distinct and large as the outer metacarpal tubercle, and is located medially (Fig. 2). Males of *Leptophryne cruentata* have an elongate and medially located inner metacarpal tubercle, but this is less distinct and noticeably smaller than the outer metacarpal tubercle, whereas *Rentapia* and *Pelophryne* lack the inner metacarpal tubercle altogether. Fingertips three and four of the new genus are truncated, reflecting arboreality as in *Pelophryne*, *Sabahphrynus*, *Rentapia*, and some species of *Ansonia*.

Etymology.—The generic name is derived from the name given by the indigenous Batak people of the Toba region in Sumatera Utara to life-sized wooden puppets called *Sigale Gale*. These puppets are used during the *papurpur sepata* funerary festivals to placate the spirits of the dead who have left no children behind. The suffix is derived from the masculine and Latinized Greek noun for toad, *phrynos*. The new genus, with a relatively large size compared with most arboreal toads in the region, lanky hands, and a wood-brown complexion, is evocative of the *Sigale Gale*.

Common name.—Puppet Toads.

Content.—Our phylogenetic analyses indicate the presence of two species within the new genus: *S. mandailinguensis* Smart et al.; and *S. minangkabauensis* Smart et al.

DESCRIPTIONS OF NEW SPECIES

Sigalegalephrynus ***mandailinguensis*** sp. nov.

Holotype.—Museum Zoologicum Bogoriense Amphibian Collection, MZB 25736 (field number ENS 15697; Fig. 3), an adult male from above the village of Sibanggor Tonga on the northeast slope of Gunung Sorikmarapi, Kecamatan Panyabungan Selatan, Kabupaten Mandailing Natal, Provinsi Sumatera Utara, Indonesia, 0.70164°N, 99.55262°E, 1383 m (in all cases, datum = WGS84), collected by U. Smart, I. Sidik, and E.N. Smith on 25 January 2014.

Paratypes (3).—UTA 63561 (field number ENS 16709), adult male from trail between the Tano Bato to Sapo Tinjak road and Lake Saba Begu, Batang Gadis National Park, Kecamatan Batang Natal, Kabupaten Mandailing Natal, Provinsi Sumatera Utara, Sumatra, Indonesia 0.70845°N, 99.51899°E at 1299 m. UTA 63562 (field number ENS 16936) and MZB 25737 (field number ENS 16937), adult males from the same locality as previous paratype but at 1297 m 0.70866°N, 99.51953°E. All paratypes collected by U. Smart, S. Handayani, and I. Sidik on 26 January 2014.

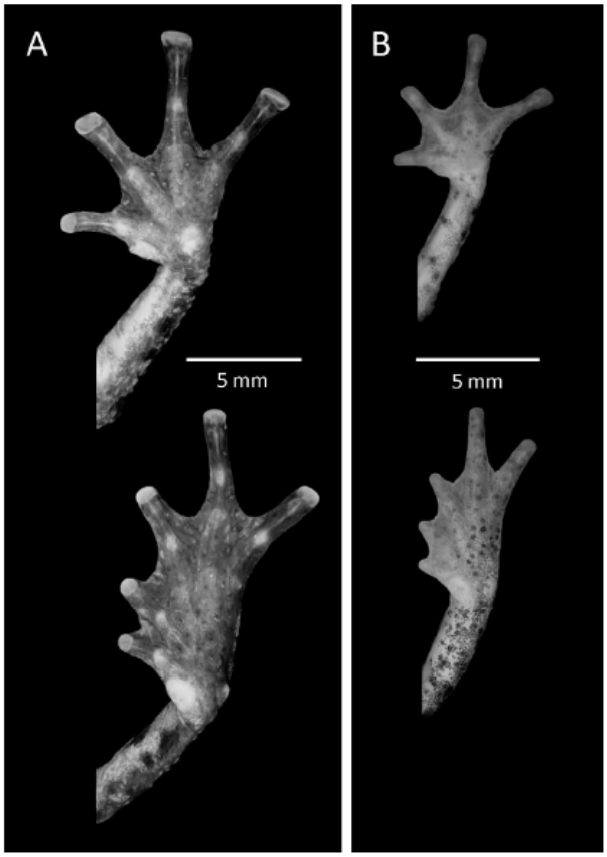

FIG. 2.—Palmar (upper) and plantar (lower) surfaces of *Sigalegalephrynus mandailinguensis* (A; MZB 25736) and *S. minangkabauensis* (B; MZB 25738).

Diagnosis.—The following combination of characters is unique to *Sigalegalephrynus mandailinguensis:* (1) This medium-sized (males 30.6–38.0 mm SVL) slender toad lacks parotoid glands. (2) The tympanum is visible. (3) The nares are closer to the tip of the snout than to the eye. (4) The fingertips are truncated and expanded. (5) The toe tips are truncated but not expanded. (6) The webbing is rudimentary in the hands and moderate in the feet. (7) The dorsal coloration consists of white and brown with a thin stripe extending from the tip of the snout to the vent at midline. (8) A dark band above orbits is joined medially, and extends as an interrupted medial track to the sacrum. (9) Alternate black–dark brown and white marks on the upper lip. (10) The flanks have a stroke of brown extending from the orbit to the inguinal area. (11) The dorsal surface is moderately tuberculate. (12) The surface of the abdomen is uniformly tuberculate, with small, smooth, and round tubercles.

Description of holotype and variation.—Holotype (adult male) followed by variation of three adult male paratypes in parentheses (UTA 63561; UTA 63562; MZB 25737). The specimen has SVL of 38.0 mm (30.6, 32.29, 32.75); head length 11.34 mm (9.5, 9.73, 10.19); head width 11.3 mm (9.65, 10.27, 10.22); snout length 5.0 mm (4.0, 3.6, 3.5); internarial distance 3.0 mm (2.5, 2.68, 2.75); eye length

FIG. 3.—(A) Lateral, (B) dorsal, and (C) ventral view of the adult male holotype of *Sigalegalephrynus mandailinguensis* from Gunung Sorikmarapi, Sumatera Utara Province, Sumatra (MZB 25736). A color version of this figure is available online.

3.3 mm (2.8, 3.1, 3); interocular distance 4 mm (3.5, 3.5, 3.7); intercanthal distance 5.7 mm (4.4, 5.0, 5.5); tympanum width 2.1 mm (1.5, 1.8, 1.8); hand length 11.2 mm (9.4, 9.1, 9.8); forearm length 11.5 mm (10, 9.6, 10.3); femur length 15.85 mm (13.46, 12.88, 13.38); tibia length 17.0 mm (13.86, 13.1, 14.02); tarsus length 9.0 mm (7.3, 7.2, 7.5); foot length 16.37 mm (13.58, 13.04, 13.68); width of fingertip pads for Finger I 0.10 mm (0.09, 0.08, 0.09), Finger II 0.13 mm (0.11, 0.10, 0.11), Finger III 0.14 mm (0.11, 0.11, 0.13), Finger IV 0.14 mm (0.10, 0.11, 0.13); width of toe pads for Toe I 0.09 mm (0.07, 0.07, 0.07), Toe II 0.09 mm (0.08, 0.08, 0.08), Toe III 0.11 mm (0.08, 0.08, 0.09), Toe IV 0.12 mm (0.09, 0.09, 0.10), Toe V 0.12 mm (0.09, 0.09, 0.11).

Body slender; head almost as long as wide; head length 30% (31%, 30%, 31%) of SVL; head width 30.0% (32%, 32%, 31%) of SVL; snout length 13% (13%, 11%, 11%) of SVL; *canthus rostralis* concave; loreal area slightly tuberculate and concave; eye length 8.7% of SVL; pupil horizontal; snout slightly sloping back toward mouth; snout mucronate and with prominent median keel, in dorsal view; tympanum distinct, with moderately developed supratympanic fold; interorbital space flat; cranial crests absent; no teeth in jaws; tongue tip oval-shaped and longer than wide; skin of dorsal

surfaces rough to finely shagreen with few large, scattered tubercles; tubercles small, rounded, and almost without keratinization; no dorsolateral, paravertebral, or occipital folds; skin on venter smooth with very fine warts; forearm length 30.3% (32.7%, 29.7%, 31.5%) of SVL; hand length 29.5% (30.7%, 28.2%, 29.9%) of SVL; relative length of Finger I < II < IV < III; fingers bearing large, expanded pads; webbing formula for hand: I$1^{3}/_{4}$–2II$1^{3}/_{4}$–$2^{1}/_{2}$III$2^{1}/_{3}$–2IV (I[2–2], [2–2], [$1^{3}/_{4}$–2] II[$1^{3}/_{4}$–$2^{1}/_{2}$], [$1^{3}/_{4}$–$2^{1}/_{2}$], [$1^{3}/_{4}$–$2^{1}/_{2}$]III[$2^{1}/_{5}$–$2^{1}/_{5}$], [$2^{3}/_{4}$–2], [$2^{1}/_{2}$–$2^{1}/_{4}$]IV); skin of forearm with tubercles; Finger I with elongate inner metacarpal tubercle, as large as outer metacarpal tubercle; each finger with one poorly developed round subarticular tubercle; nuptial excrescence brownish-dark, glandular, and dorsomedially extended with keratinized spicules present at the base of Finger I; femur length 41.7% (44%, 39.9%, 40.9%) of SVL; tibia length 44.8% (45.3%, 40.6%, 42.8%) of SVL; tarsal length 23.7% (23.9%, 22.3%, 22.9%) of SVL; foot length 43.1% (44.4%, 40.4%, 41.8%) of SVL; relative lengths of toes I < II < III < V < IV; toes bearing large pads; feet with moderate webbing, webbing formula for the feet: I0–$1^{1}/_{3}$II0–$1^{3}/_{4}$III2–$2^{1}/_{5}$IV$2^{1}/_{5}$–$1^{2}/_{3}$V (I[0–1], [1–$1^{1}/_{2}$], [0–$1^{1}/_{4}$]II[1–2], [1–2], [1–2]III[$1^{1}/_{3}$–3], [$1^{1}/_{2}$–$2^{1}/_{3}$], [1–$2^{3}/_{4}$]IV[$2^{2}/_{3}$–2], [$2^{2}/_{3}$–$1^{2}/_{3}$], [$2^{2}/_{3}$–$1^{3}/_{4}$]V); heels without tubercles; inner metatarsal tubercle moderately developed and elongate; outer metatarsal tubercle absent; one moderate subarticular tubercle present at the base of first phalanx on each toe; toes without toe pads.

Color in life.—Distinct light brown spot on each side of lore and distinct whitish spot on upper jaw just below posterior end of orbit; dorsum brown with light chamois-brown-colored hourglass marking; distinct darker spots dorsolateral of sacral and iliac joint; alternate black and brown bands on dorsal side of limbs; in lateral flanks, skin with distinct blackish stripe extending from posterior end of eyes through top of humeral–ulnar joint to inguinal area; short, black dorsal stripe on each side of pubic junction; venter yellowish-white maculated with black blotches; chest and ventral surface of limbs light tea-rose orange with few sparsely located yellowish dots; tips of fingers and toes tea-rose orange-colored without dots; iris bright gold with black reticulations.

Color in preservative.—In preservative the color of the animal differs slightly from that in life. The dorsum has a very light hourglass marking. The venter is whitish grey maculated with dark brown blotches.

Etymology.—The specific epithet is an adjective referring to the Mandailing Batak ethnic region and Kabupaten (regency) where the new species was found.

Common name.—Mandailing Puppet Toads.

Distribution and natural history.—*Sigalegalephrynus mandailinguensis* is known only from rainforests on the slopes of Gunung Sorikmarapi in southern Sumatera Utara from 1297 m to 1383 m (Figs. 4 and 5). The holotype was found at 1830 h inactive on a cave wall, ~2 m from the entrance and below ground level. The paratypes were active on vegetation 0.5 m, 2 m, and 3 m (on a liana) above ground between 1930 and 2115 h. The two individuals higher above the ground were vocalizing using a series of high-pitched ticks. The holotype weighed 2.5 g, and the paratypes 1.4, 1.7, and 1.8 g.

Call.—The advertisement call of Sigalegalephrynus mandailinguensis is best described as a rapid succession of regularly placed, shrill ticks. A single call 17.27 s in length was recorded at 2156 h. The call begins with infrequent, low-amplitude ticks, which progressively get louder and attain a uniform pulse rate during the main calling period that, in our recording, lasted ~15 s. During this time, the tonal pulses were organized in a discreet series repeated at a moderately high rate of 6 times/s. Each individual pulse lasted about 29 ± 4 ms (Fig. 6) intersected by pauses ~12 ± 9 ms long. These pulses were relatively narrow band (<500 Hz). The average dominant frequency of pulses was 3.4 ± 0.04 Hz.

Sigalegalephrynus ***minangkabauensis*** sp. nov.

Holotype.—Museum Zoologicum Bogoriense Amphibian Collection, MZB 25738 (field number ENS 16028), an adult male (Fig. 7) from Gunung Kunyit, Kecamatan Panyabungan Selatan, Kabupaten Kerinci, Provinsi Jambi, Indonesia, 2.26013°S, 101.49512°E at 1402 m, collected by E. Wostl, E.N. Smith, W. Trilaksono, and G. Barraza on 24 June 2013.

Diagnosis and comparison.—The following combination of characters is unique to *Sigalegalephrynus minangkabauensis:* (1) A small (19.32 mm SVL) and slender toad without parotoid glands. (2) Fingertips I and II are rounded and not expanded. (3) Fingertips III and IV are rounded and expanded. (4) The toe tips are rounded but not expanded. (5) The webbing is rudimentary in the hands and moderate in the feet. (6) The dorsum is light greenish-brown with a middorsal pinstripe extending from the tip of the snout to the vent. (7) The flanks have a single stroke of dark brown extending from the posterior end of the orbit to the inguinal region. (8) The dorsal surface is moderately tuberculate. (9) The ventral surface is smooth with scattered black spots.

Sigalegalephrynus minangkabauensis can be distinguished from *S. mandailinguensis* (characters in parentheses) based on the following differences. The tympanum is barely discernible (tympanum distinct). The finger-pads are moderately defined (finger-pads prominent). The fingertips are rounded but not expanded (distinctly spatulate on tips III to IV). The hands lack subarticular tubercles (distinct subarticular tubercles under Fingers III and IV). The webbing of the foot is more extensive, extending to the last phalanx on Toes I and II (last phalanx free of webbing). The pads on toes are moderately defined (pads prominent). The feet lack subarticular tubercles (distinct subarticular tubercles under Toes IV and V). The overall texture is glossy with fewer tubercles on the dorsum and flanks (overall texture rugose, body and flanks extensively tuberculate). Taking into account the uncorrected genetic distance between the two species of *Sigalegalephrynus* (Table 2), the aforementioned comparisons provide adequate diagnostic characters to warrant *S. minangkabauensis* as a species distinct from *S. mandailinguensis*.

Description of holotype.—The holotype (sex indistinguishable) has SVL of 19.32 mm; head length 6.94 mm; head width 6.57 mm; snout length 2.7 mm; eye length 2.2 mm; eye–nares length 1.6 mm; distance between nares to tip of snout 0.6 mm; internarial distance 1.8 mm; intercanthal distance 3.6 mm; forearm length 6.0 mm; hand length 5.5 mm; femur length 8.5 mm; tibia length 8.71 mm; tarsal

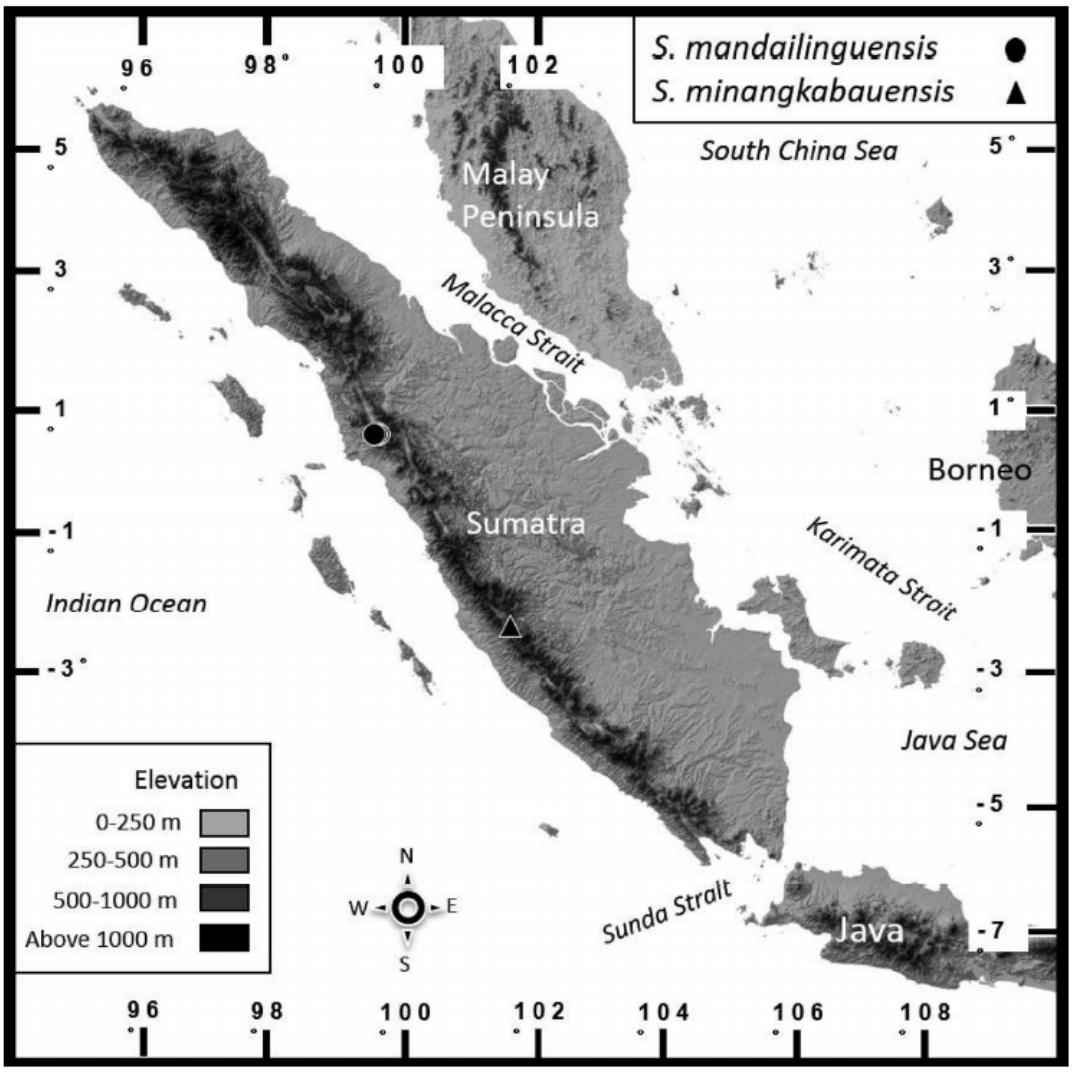

FIG. 4.—Map of Sumatra with topographical relief showing localities where members of the new genus of toad (*Sigalegalephrynus*) were found.

length 5.1 mm; foot length 7.5 mm; width of fingertip pad of Finger III 1.6 mm; Finger IV 1.4 mm.

Body slender, head little longer than wide; head length 36.1% SVL, head width 34.0% SVL; snout length 14% SVL; *canthus rostralis* concave; loreal area without tubercules and concave; snout truncated and slightly sloping back toward mouth; snout mucronate and with prominent median keel in dorsal view; eye length 11.4% SVL; pupil horizontal; upper eyelid granular; tympanum barely visible, with no supratympanic fold; interorbital space flat; cranial crests absent; no teeth in jaws; tongue tip oval-shaped, longer than wide; skin of dorsal surfaces rough to finely shagreen with few large, scattered tubercles; tubercles small, rounded, and almost without keratinization; no dorsolateral, paravertebral, or occipital folds; skin on venter smooth with few fine warts; forearm length 31.1% SVL; hand length 28.5% SVL; relative lengths of Fingers I < II < IV < III; fingers bearing expanded pads; webbing of hands moderate: between Fingers I and II reaches distal and proximal subarticular tubercles, respectively; between Fingers II and III reaches proximal subarticular tubercles; between Fingers III and IV reaches proximal subarticular tubercles; webbing formula for the hand $I1^{1}/_{2}$–2II2–$2^{2}/_{3}$III$2^{2}/_{3}$–2IV; elongate inner metacarpal tubercle below Finger I as large as outer metarcarpal tubercle; lower arm with indistinct tubercles; fingers without expanded pads; femur length 44.0% SVL; tibia length 45.0% SVL; tarsal length 26.3% SVL; foot length 38.8% SVL; relative lengths of toes I < II < III V< IV; heels without tubercles; inner metatarsal tubercle weakly developed and elongate; outer metatarsal tubercle absent; webbing formula for the feet: I0–0II0–2III1–3IV$2^{3}/_{4}$–2V; no expanded pads on toes.

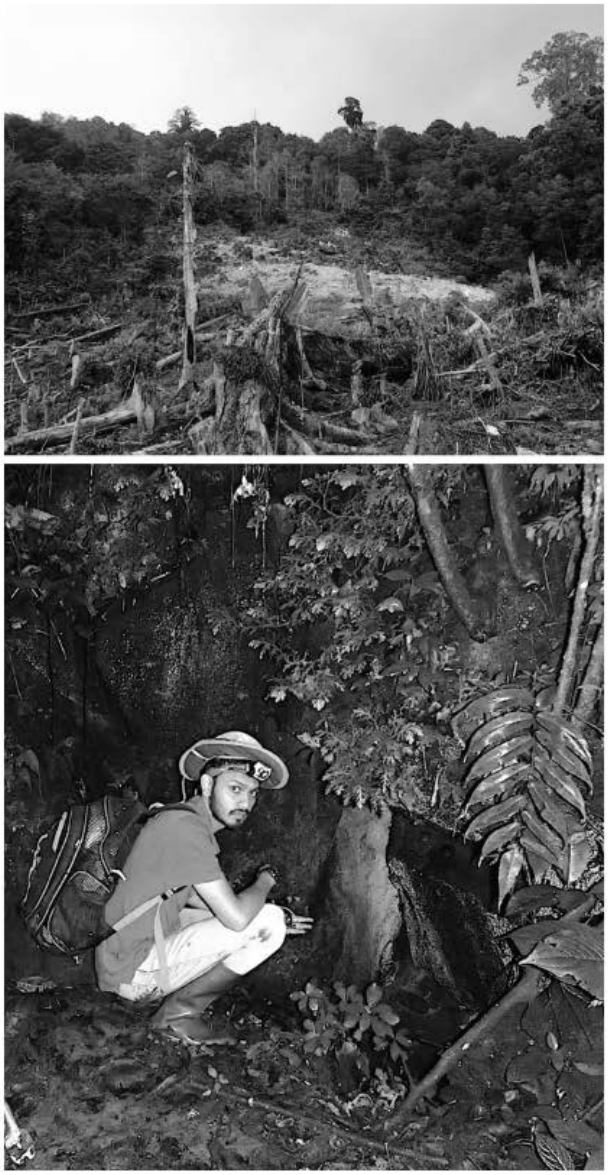

FIG. 5.—Habitat of *Sigalegalephrynus mandailinguensis*—a view of the rainforest at the edge of an inactive solphatara field on the northeastern slope of Gunung Sorikmarapi where the holotype was found (upper); and first author at the entrance of the subterranean hollow where the holotype was collected (lower).

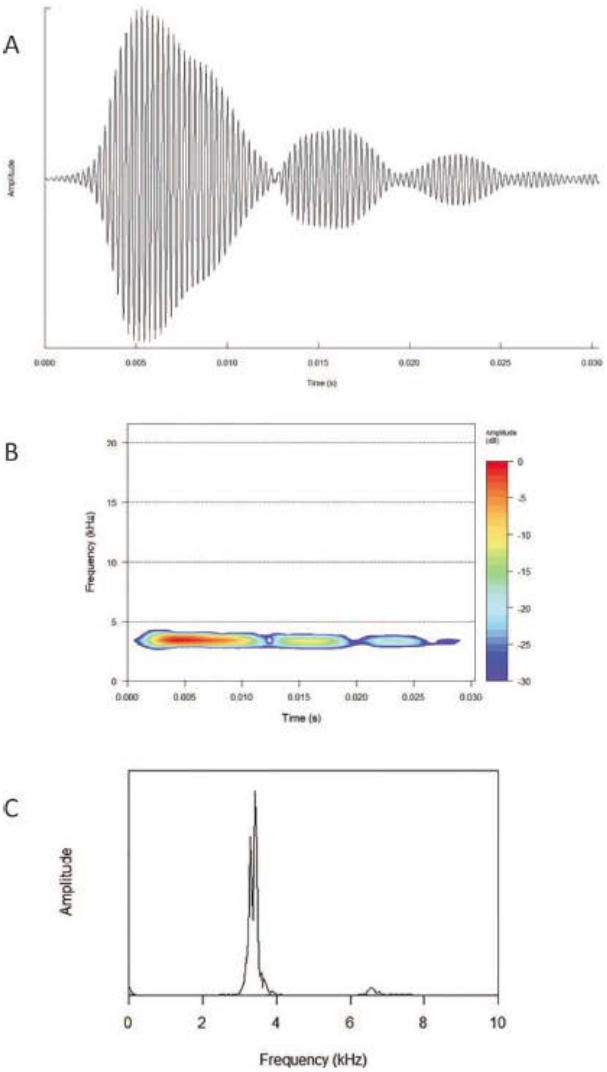

FIG. 6.—Advertisement call of *Sigalegalephrynus mandailinguensis*. (A) oscillogram (relative amplitude vs. time) and (B) spectrograms (energy in each frequency vs. time) of a single pulse; (C) power spectra. A color version of this figure is available online.

Color in life.—Edges of lore and head golden with black shades; area below eyes with prominent white marking with yellowish tint; dorsum light greenish-brown with light brown hourglass figure extending from posterior of orbit to top of the sacroiliac joint; hourglass shape ends with distinct horizontal black bean color mark on each side; yellowish-green marking on each shoulder; flanks black with red tubercles, maculated with greenish-yellow blotches, and possessing very prominent dark brown stripe starting from posterior end of orbit to inguinal region; inguinal areas greenish with golden tint; sacroiliac joint to inguinal region of flanks, golden yellowish-green; dorsal sides of limbs light brown; forearm, femur, tibia–fibula, and tarsus with distinct dark spot encircled with golden-yellowish-green color; venter opaque; throat golden-yellow; abdomen, ventral side of arms and legs pinkish with scattered yellow and black blotches; webbing in hand and foot translucent.

Color in preservative.—In preservative, the animal appears dull because it has lost its golden-yellowish and greenish colors. The hourglass pattern has turned gray. The venter has lost all of its pinkish and golden-yellowish shades and turned a greenish-white.

Etymology.—The specific epithet refers to the Minangkabau or Minang ethnic group inhabiting the region where the new species was found.

Common name.—Minangkabau Puppet Toads.

FIG. 7.—(A) Lateral, (B) dorsal, and (C) ventral view of the holotype of *Sigalegalephrynus minangkabauensis* from Gunung Kunyit, Sumatera Utara Province, Sumatra (MZB 25738). A color version of this figure is available online.

Distribution and natural history.—*Sigalegalephrynus minangkabauensis* is known only from Gunung Kunyit from an elevation of 1428 m (Fig. 4). The holotype was found perched on a leaf ~1.25 m above ground, by the edge of a forest stream at 2015 h. Before collecting it, ENS watched the specimen move in reverse toward the edge of the leaf on which it was perched, where it defecated (outside of the surface of the leaf), and then return to its original position. The holotype weighed 0.5 g.

DISCUSSION

Advertisement Calls

There is limited information on the acoustic properties of advertisement calls for toads of Sundaland. Nevertheless, a comparison with call data collected in previous field expeditions and available information shows that the rapid pulse of metallic ticks vocalized by *Sigalegalephrynus mandailinguensis* is unique in its structure and spectral properties among calls made by bufonids in the Sunda Shelf.

The advertisement call of *Duttaphrynus melanostictus*, for example, is a shrill, gritty chatter that has been described as a long train of pulses repeated at a regular rate, with an average dominant frequency of 1293 kHz (Ngo and Ngo 2013). *Ansonia leptopus* calls have a varying range of dominant frequency and, unlike *S. mandailinguensis*, consist of two distinct pulses, one short and one long (Matsui 1982). *Phrynoidis asper* advertisement calls are typically a three-note repertoire consisting of a high-pitched "bock" followed by a couple of deep, raspy honks. The call of *Leptophryne borbonica* usually consists of croaky crooning that is reminiscent of marbles rubbing against each other. *Ingerophrynus biporcatus* calls are best described as a brisk series of guttural bleats, whereas those of *Pseudobufo subasper* consist of a volley of squeaky chirps. The pulses of the Indian endemic *Pedostibes tuberculosus* have been described as rapid series of *schirr schirr*, with a dominant frequency of 3782.13 kHz (Gururaja and Ramachandra 2006).

Phylogeny of Sunda Shelf Toad Genera

The systematics of Southeast Asian bufonids at the generic level has been challenging, and the recent molecular phylogenetic attempts at clarifying higher lever relationships have generally failed to resolve relationships (Frost et al. 2006; Matsui et al. 2007, 2010; Van Bocxlaer et al. 2009, 2010). Consequently, the taxonomy of Southeast Asian toad genera has been in flux (e.g., Chandramouli and Amarasinghe 2016).

Based on existing data, this lack of phylogenetic resolution appears to stem primarily from the presence of short internal braches. A phylogeny showing long branches interspersed within a backbone of short internal branch lengths is usually indicative of rapid radiations and is known to present a significant challenge for phylogenetic inference (Whitfield and Lockhart 2007; Whitfield and Kjer 2008; Rothfels et al. 2012). According to several phylogenetic studies (e.g., Pramuk et al. 2008; Van Bocxlaer et al. 2010), the early evolutionary history of bufonids involved an episode of rapid expansion and radiation out of South America that allowed for a nearly cosmopolitan distribution in a very short time. This could explain the difficulty in achieving clearer resolution for the deeper nodes in the phylogeny of South and Southeast Asian toad genera. However, short internal nodes could also be symptomatic of inadequate sampling, disagreement within or among data sets, or loss of phylogenetic signal over time (Whitfield and Lockhart 2007; Whitfield and Kjer 2008), in which case, a more thorough sampling of taxonomic diversity and selection of more informative loci would provide better resolution for this clade. The relative influence of the aforementioned factors should be addressed prior to making any additional attempts at inferring the higher level relationships of Sundaland bufonids.

Although patterns of the early diversification of Sundaland bufonids remain poorly resolved in our study, the phylogeny recovered by our analyses is, for the most part, congruent with previous studies (Frost et al. 2006; Matsui et al. 2007; Van Bocxlaer et al. 2009, 2010; Pyron and Wiens 2011). Ours is the first study to incorporate the Bleeding Toads (*Leptophryne cruentata*), whose generic affinity to *L. borbonica*, as designated by morphology (Fitzinger 1843), is upheld by our molecular results. Ours is also the first study to include the unique aquatic toads (*Pseudobufo subasper*) in a phylogenetic assessment. The relationship of a *Duttaphrynus melanostictus* specimen from Sumatra with *Pedostibes tuberculosus* agrees with Van Bocxlaer et al. (2009), who suggested *Duttaphrynus* formed the sister taxon to *Xanthophryne*, an Indian endemic not included in our study. This appears to indicate an Indian origin for *Duttaphrynus*, with a recent invasion into Southeast Asia. The lack of higher level phylogenetic resolution, however, limits any attempt at a biogeographic appraisal of these toads. In the context of the nonmonophyly of *Pedostibes*, our results agree with Chan et al. (2016).

Based on the most comprehensive representation of Sundaland toad genera to date, our results provide a phylogenetic framework for future systematic and taxonomic studies. More importantly, our analyses using both maximum likelihood and Bayesian inference show the new taxa to be phylogenetically isolated from all other genera with high support, presenting a strong justification for the recognition of a new genus.

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ABSTRAK (INDONESIAN): Kami mendeskripsikan satu marga baru dan dua jenis baru kodok dari wilayah gunung api di Provinsi Sumatera Utara (Gunung Sorik Merapi) dan Provinsi Jambi (Gunung Kunyit). Takson baru ini dapat dibedakan satu sama lain maupun dengan marga kodok lainnya berdasarkan perbedaan genetik, morfologi, dan struktur suara panggilan kodok tersebut. Kami menggunakan data DNA mitokondria dan DNA inti kodok-kodok dari famili Bufonidae yang terdapat di wilayah Paparan Sunda untuk membuat hipotesis filogenetik mengenai hubungan kekerabatan antar marga kodok tersebut. Secara umum, hasil penelitian yang kami peroleh mendukung hasil penelitian-penelitian sebelumnya. Di samping itu, kami juga menemukan untuk pertama kalinya bahwa posisi filogenetik *Pseudobufo* dan marga baru berada di bagian paling dasar dari seluruh marga katak di daerah Paparan Sunda, dengan pengecualian pada marga *Duttaphrynus*.

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Appendix

Specimens Examined

Ansonia glandulosa ($n = 1$).—INDONESIA: Sumatera Selatan: MZB 4239 (male).

Ansonia latidisca (*n* = 1).—INDONESIA: Kalimantan Barat: Mount Damus, Sambas, RMNH 10677–Holotype.

Ansonia spinulifer (*n* = 2).—INDONESIA: Kalimantan: Kabupaten Singkawang: UTA 62129 (female), UTA 62130 (male).

Duttaphrynus melanostictus (*n* = 11).—INDONESIA: Jawa Barat: Kabupaten Bandung: Gunung Kencana, 1751 m, 7.28596°S, 107.63842°E, UTA 63437 (female); Cicayur, 839 m, 7.30199°S, 107.57725°E,UTA 63438 (male); road from Pangelengan to Sri Kandi, 1557 m, 7.25889°S, 107.61554°E, UTA 63439 (male); Kabupaten Bogor: Desa Sukamaki, P.T. Vivaria indonesia, Frog Farm, 665 m, 6.669833°S, 106.957833°E, UTA 53738 (female); 6.669833°S, 106.957833°E, UTA 53739 (male); Taman Safari, 1115 m, 6.726000°S, 106.950833°N, UTA 53740 (female) Hotel Wisma Tamu, Kampus IPB, 192 m, 6.55556°S, 106.72697°E, UTA 62527 (male); Jawa Timur: Kabupaten Malang: Between Ngasem and Ngadilangkung, SE foothills of Gunung Butak, 359 m, 8.09481°S, 112.56774°E, UTA 62528 (male); road from Kraton Gunung Kawi to Malang, 360 m, 8.09644°S, 112.5373°E, UTA 62530 (female); Lampung: Kabupaten Lampung Selatan: Gunung Rajabasa: S slope, 150–250 m, 5.804833°S, 105.622833°E, UTA 53746 (female); Kepulauan Riau: Bintan Island: RMNH 3961–Lectotype (male).

Ingerophrynus biporcatus (*n* = 11).—INDONESIA: Jawa Barat: Kabupaten Bogor: Kampus IPB pond, 225 m, 6.55877°S, 106.72076°E, UTA 63768; Lampung: Kabupaten Lampung Selatan: Gunung RajabasPanpul Sukaraja Trail, 170 m, UTA 53731 (male); south slope Gunung Rajabasa, Desa Canti, 70 m, 5.80937°S, 105.59759°E, UTA 63400 (female); 102 m, 5.80791°S, 105.60078°E, UTA A 63399 (female); 122 m, 5.80164°S, 105.59772°E, UTA 63401 (female); 31 m, 5.80613°S, 105.59599°E, UTA 63402 (female); Kabupaten Pasawaran: Margadalom, Padang Cermin, 10 m, 5.554167°S, 105.185833°E, UTA 53730; Hill's North of Padang Cermin, 259 m, 5.52335°S, 105.18996°E, UTA 63403 (female); Gunung Pesawaran, 936 m, 5.51791°S, 105.0731°E, UTA 63406 (male); Kabupaten Tanggamus: Vicinity of Ngarip Geothermal Plant, 777 m, 5.32536°S, 104.57709°E, UTA 63404 (male); Hill Above Ngarip, 1233 m, UTA 63405 (male).

Ingerophrynus claviger (*n* = 3).—INDONESIA: Sumatera Selatan: Unknown: Road from Pagar Alam to Manna, 593 m, 4.18527°S, 103.08159°E, UTA 63770 (male), UTA 637701 (male), UTA 637702 (male).

Ingerophrynus parvus (*n* = 1).—INDONESIA: Sumatera Utara: Kabupaten Deli Serdang: "Boy Scout Camp" in Bandar Baru, 904 m, 3.26835°N, 98.53953°E, UTA 63769 (male).

Leptophryne borbonica (*n* = 16).—INDONESIA: Bengkulu: Unknown: MZB 2931; MZB 3479 (female); MZB 3554 (male); MZB 9337 (male); MZB 16312 (female); Jambi: Unknown: MZB 21845 (male); Jawa: Unknown: MZB 23838 (male); MZB 6716 (female); MZB 6717 (male); MZB 6719 (female); RMNH 1739–Syntype; Jawa Barat: MZB 15155 (female); Kalimantan: Unknown: MZB 3641 (male); Lampung: Kabupaten Tanggamus: MZB 3445 (female); hill above Ngarip, 5.28218°S, 104.55773°E, UTA 62486; Sumatera Selatan: Unknown: MZB 4234 (female).

Leptophryne cruentata (*n* = 2).—INDONESIA: Jawa Barat: Kabupaten Cianjur: Gunung Gede Pangrango National Park, 1421 m, 6.74345°S, 107.00355°E, UTA 62522; Cibodas, RMNH 2130–Syntype.

Pelophryne brevipes (*n* = 6).—INDONESIA: Jambi: MZB 14950 (female); Lampung: Kabupaten Pesawaran: Gunung Pesawaran, 1046 m, 5.51563°S, 105.07667°E, UTA 63762; Unknown: MZB 14607; Sumatera Barat: MZB 3185 (male); Sumatera Selatan: MZB 12078 (female); Unknown: Unknown: MZB 17097 (female).

Pelophryne signata (*n* = 6).—INDONESIA: Jambi: MZB 21.864 (female); MZB 21.863; Kalimantan Barat: MZB 4342 (male); MZB 4343 (male); MZB 4344 (female); Kalimantan Timur: MZB 6283 (female).

Phrynoides asper (*n* = 10).—INDONESIA: Jawa Barat: Kabupaten Bogor: Desa Sukamaki P.T. Vivaria indonesia, Frog Farm, 660 m, 6.669833°S, 106.957833°E, UTA 53719; Lampung: Kabupaten Lampung Barat: Kubuperahu, 730 m, 5.06467°S, 104.03623°E, UTA 62469 (female); Kabupaten Pasawaran: Road between Padang Cermin and Kedongdong, 382 m, 5.54653°S, 105.04678°E, UTA 61449 (male); road between Padang Cermin and Kedongdong 454 m, 5.55196°S 105.06131°E, UTA 62448 (male); road between Padang Cermin and Kedongdong 189 m, 5.48787°S, 105.01685°E, UTA 62453 (male), UTA 62454 (male); Kabupaten Tanggamus: Ngarip paddy fields, 869 m, 5.30638°S, 104.54827°E, UTA 62459 (male); forest above Ngarip Town, 1324 m, 5.2822°S, 104.55693°E, UTA 62463 (female); NE of town of Ngarip, 830 m, 5.31446°S, 104.54094°E, UTA 62467; Unknown: Unknown: RMNH 2172–Lectotype.

Phrynoides juxtasper (*n* = 9).—INDONESIA: Jambi: Kabupaten Kerinci: beginning of trail to Danau Tujuh, 1409 m, 2.04139°S, 101.31462°E, UTA 62414 (female); trail to Danau Tujuh, 1565 m, 1.70868°S, 101.36981°E, UTA 62415 (male); Unknown: Unknown: Road between Tapan and Sungaipenuh, 30 km W of Sungaipenuh, UTA 63767 (male); Jawa Barat: Kabupaten Bogor: Desa Sukamaki P.T. Vivaria Indonesia, Frog Farm, 660 m, 6.672000°S, 106.879667°E, UTA 53719 (female); Sumatera Utara: Kabupaten Deli Serdang: Boy Scout Camp in Bandar Baru, 901 m, 3.26898°N, 98.53992°E, UTA 63431 (male); Boy Scout Camp in Bandar Baru, 906 m, 3.26787°N, 98.53967°E, UTA 63432 (female); Kabupaten Samosir: W coast of Island S of Pangururan, 926 m, 2.45681°N, 98.8438°E, UTA 63434 (male); Kabupaten Mandailing Natal: Kota Baringen Julu (Batang Gadis National Park), 1174 m, 0.66608°N, 99.57893°E, UTA 63436 (female); Kalimantan Timur: Unknown: Upper Mahakam River, RMNH 14517–Paratype.

Pseudobufo subasper (*n* = 4).—INDONESIA: Riau: Sungai Kerumutan, UTA 63763 (female); UTA 63764 (male); Unknown: Unknown: RMNH 2199–Syntype (male); RMNH 2200–Syntype (female); RMNH 2216–Syntype.

Rentapia hosii (*n* = 9).—UNKNOWN: Unknown: Unknown: UTA 38040 (female); UTA 43516 (female); UTA 6395 (female); UTA 6396 (female); UTA 6430 (female); UTA 6436 (female); UTA 6494 (male); UTA 6520 (male); UTA 6705 (female).

Rentapia rugosa (*n* = 1).—MALAYSIA: Sarawak: MZB 8401.

Sigalegalephrynus mandailinguensis (*n* = 4).—INDONESIA: Sumatera Utara: Kabupaten Mandailing Natal: Gunung Sorikmarapi (W side), 1383 m, 0.70164°N, 99.55262°E, MZB 25736–Holotype (male); trail between the Tano Bato to Sapo Tinjak road and Lake Saba Begu, Batang Gadis National Park, 1297 m 0.70866°N, 99.51953°E, MZB 25737–Paratype (male); 1299 m, 0.70866°N, 99.51953°E, UTA 63562–Paratype (male), UTA 63561–Paratype (male).

Sigalegalephrynus minangkabauensis (*n* = 1).—INDONESIA: Jambi: Kabupaten Kerinci: Gunung Kunyit, 1428 m, 2.26013°S, 101.49512°E, MZB 25738–Holotype.

CHAPTER THREE

Multilocus phylogeny of the Puppet Toads from Sumatra, Indonesia (Anura: Bufonidae: *Sigalegalephrynus*), and description of a new species with implication for conservation

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ABSTRACT

The puppet toads of Sumatra, *Sigalegalephrynus*, belong to one of the three least known bufonid genera in South and Southeast Asia and are critical to our understanding of toad diversity in these regions. We assessed phylogenetic relationships of the puppet toads of Sumatra using mitochondrial (*12S* rRNA and *16S* rRNA genes with flanking $tRNA^{Val}$ gene) and nuclear (*NCX1* and *POMC* genes) DNA markers. Phylogenetic analyses of both mitochondrial and nuclear molecular data recovered six distinct species lineages, including one previously undetected and nested within the northern clade of *Sigalegalephrynus*. Surprisingly, though the new species lineage occurs on the same mountain as *S. burnitelongensis*, it is genetically more similar to *S. gayoluesensis* (8.0% and 6.3% *16S* gene resemblance, respectively). Both maximum likelihood and Bayesian analyses of the concatenated mtDNA and nuDNA data revealed identical topologies for *Sigalegalephrynus*, placing the new lineage as the basal taxon for both *S. gayoluesensis* and *S. burnitelongensis*. This lineage is also morphologically distinct from its congeners. We, therefore, propose the new lineage as the candidate of a new *Sigalegalephrynus* species, and present and compare osteological data from micro-CT scanning for the three *Sigalegalephrynus* species of the northern clade.

Key words: Computed tomography (CT), Indonesia, IUCN Red List, *Sigalegalephrynus* sp. nov., Southeast Asia, Sunda Shelf

INTRODUCTION

Sigalegalephrynus Smart, Sarker, Arifin, Harvey, Sidik, Hamidy, Kurniawan & Smith 2017 is a mysterious and very interesting toad genus endemic to Sumatra (Smart *et al.* 2017, Chan & Grismer 2019, Sarker *et al.* 2019). Based on previous studies by Smart *et al.* (2017) and Chan & Grismer (2019), this genus is basal to all Southeast Asian bufonid genera. *Sigalegalephrynus* contains five species, *S. mandailinguensis*, *S. minangkabauensis*, *S. burnitelongensis*, *S. gayoluesensis*, and *S. harveyi* (Sarker *et al.* 2019). With the exception of *S. minangkabauensis*, described from a single juvenile, all species are described based on at least two adult individuals. All known *Sigalegalephrynus* are highland species occurring between 1299 and 1878 m on isolated mountaintops. *Sigalegalephrynus harveyi* occurs between 1826 and 1878 m on Gunung Dempo, Kabupaten Pagar Alam, Provinsi Sumatera Selatan; *S. minangkabauensis* at 1402 m on Gunung Kunyit, Kabupaten Kerinci, Provinsi Jambi; *S. mandailinguensis* from 1299 to 1383 m on Gunung Sorikmarapi, Batan Gadis National Park, Kabupaten Mandailing Natal, Provinsi Sumatera Utara; *S. gayoluesensis* occurs from 1796 to 1850 m on mountains near Kenyaran Pantan Cuaca, Kabupaten Gayo Lues, Provinsi Aceh; and *S. burnitelongensis* at 1519 m on Gunung Burni Telong, Kabupaten Bener Meriah, Provinsi Aceh. So far, no species are known to occur in sympatry (Sarker *et al*. 2019). All of the *Sigaelgaelphrynus* species have been propose to be placed under "Endangered" category of IUCN Red List category.

While revising our 2015 summer trip collection, we found a juvenile specimen of *Sigalegalephrynus* from 1734 m on Gunung Burni Telong, 200 m above in elevation and about one mile away to the northeast of the type locality of *S. burnitelongensis*. Though it is a subadult female, it is morphologically distinct from other congeneric species, including the two species currently known from Aceh Province—*S. gayoluesensis* and *S. burnitelongensis*. This specimen differs from *S. burnitelongensis* and *S. gayoluesensis*, in external and internal

morphology and in DNA sequence, and we describe it as a new species adopting the lineage-based Unified Species Concept of de Queiroz (2007).

MATERIAL AND METHODS

Sigalegalephrynus specimens were obtained in Sumatra in accordance to protocols approved by the UTA Institutional Animal Care and Use Committee (UTA IACUC A12.004). We took photographs of live specimens and immediately after euthanasia, in dorsal, ventral, and lateral aspects. Later, we took muscle or liver tissue samples and preserved them in 1.5 mL of cell lysis buffer solution (0.5 M Tris/0.25% EDTA/2.5% SDS, pH = 8.2). Specimens were then fixed in 10% formalin for 24 hours and then transferred to 70% alcohol for preservation. Finally, we deposited all specimens at the Laboratory of Herpetology in the Museum Zoologicum Bogoriense (MZB), and the Amphibian and Reptile Diversity Research Center (ARDRC) of the University of Texas, Arlington (UTA). Museum acronyms follow Sabaj Perez (2016).

Morphological data. Terminology of external characteristics follows Smart *et al.* 2017, Campbell *et al.* (2018), and Sarker *et al.* (2019). Webbing formulae follow Savage & Heyer (1967), as modified by Myers & Duellman (1982) and Savage & Heyer (1997). We used digital color photographs of the new species and followed Kok & Kalamandeen (2008) to describe color in life and other qualitative characteristics; these images are deposited at the University of Texas at Arlington digital image collection.

All external morphology measurements collected by a single observer (ENS) using digital calipers or an ocular micrometer to the nearest 0.1 mm. We measured: 1) snout–vent length (SVL)—tip of snout to anterior margin of vent; 2) head length (HL)—posterior angle of jaw to tip of snout; 3) head width (HW)—ventrally at angles of jaw, excluding warts; 4) snout length (SNL)—anterior corner of eye to snout tip; 5) intercanthal distance (ICD)—

distance between anterior edges of canthi; 6) internarial distance (IND)—distance between anterior ends of nares; 7) eye to naris distance (END)—distance from the anterior corner of eye to posterior border of naris; 8) naris to snout distance (NSD)—distance from anterior border of naris to the tip of snout; 9) interorbital distance (IOD)—minimal distance between upper eyelids; 10) UELW ; 11) tympanum length (TML)—maximum horizontal width/diameter of tympanum; 12) tympanum-eye diameter (TED)—distance from the outer edge of the tympanum to the posterior margin of the eye; 13) eye length (EL)—horizontal distance from anterior to posterior junctions of upper and lower eyelids; 14) internarial distance (IND)—distance between the proximal borders of nares; 15) forearm length (FAL)—tip of elbow to proximal margin of outer metacarpal tubercle; 16) hand length (HAL)—proximal margin of metacarpal tubercle to Finger III tip; 17) thigh length (THL)—center of cloaca to distal surface of knee, appressed; 18) tibia length (TBL)—when positioning hind limb in a Z pattern; 19) tarsus length (TRL)—tibio-tarsal articulation to proximal margin of outer metatarsal tubercle; 20) foot length (FTL)—proximal margin of outer metatarsal tubercle to Toe IV tip; 21) inner metacarpal tubercle length (IMCL)—distal to proximal end; 22) inner metacarpal tubercle width (IMCW)—at greatest width, measured perpendicularly to IMCL; 23) outer metacarpal tubercle length (OMCL)—distal to proximal end; 24) outer metacarpal tubercle width (OMCW)—at greatest width, measured perpendicularly to OMCL; 25) inner metatarsal tubercle length (IMTL)—distal to proximal end; 26) inner metatarsal tubercle width (IMTW)—at greatest width, measured perpendicularly to IMTL; 27) outer metatarsal tubercle length (OMTL)—distal to proximal end; 28) outer metatarsal tubercle width (OMTW)—at greatest width, measured perpendicularly to IMTL; and 29) length of fingers (F1L–F4L)—from tip of fingers to first phalangeal-metacarpal joint; 30) length of toes (TlL–T5L)—from tip of fingers to first phalangeal-metatarsal joint; 31) width of third finger disc (F3PD)—at right angle to digital

axis; 32) width of proximal end of penultimate phalanx of third finger (F3PB)—at right angle of digital axis.

Taxon sampling and DNA sequencing. To isolate DNA and purify PCR products, we used Serapure beads following the Agencourt protocol (Beckman Coulter Co., Fort Collins, CO, USA), after Rohland & Reich (2012). Initially, to barcode, we sequenced a fragment of the mitochondrially encoded 16S rRNA gene (*16S*) from 15 new *Sigalegalephrynus* specimens, following Smart *et al.* (2017) and Sarker *et al.* (2019) and not previously sequenced by these researchers. Finally, after an initial examination of the newly generated sequences, we used 10 primer combinations and amplified up to 2540 bp of mitochondrial *12S* and *16S* rRNA along with the flanking $tRNA^{val}$ (Goebel *et al.* 1999; Feller & Hedges 1998; Titus & Larson 1996; Hedges 1994; Palumbi *et al.* 1991; Wilkinson *et al.* 1996), nuclear *NCX1* (Bossuyt & Milinkovich 2000) and *POMC* (Wiens *et al.* 2005; Pramuk 2006) from 12 specimens. PCR success was visually assessed on a 1% agarose gel, and PCR products were purified. Sequencing reactions were completed at the Life Science Genomic Core Facility of the University of Texas at Arlington with an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Following Sarker *et al.* (2019), for initial phylogeny exploration, we used a fragment of *16S* from 54 specimens, including 35 sequences from GenBank, including at least one representative from each extant Southeast Asian bufonid genus, one New World bufonid (*Atelopus flavescens*), and one hylid frog (*Dryophytes arenicolor)* as outgroups. We then chose one representative specimen from each *Sigalegalephrynus* species as ingroup and five species from five genera—*Leptophryne* (1), *Pelophryne* (1), *Ingerophrynus* (1), *Phrynoidis* (1) and *Pseudobufo* (2), as outgroups, for additional sequencing and further exploring phylogenetic relationships using more mitochondrial sequence and nuclear genes. All

sequences generated for this project will be submitted to GenBank upon submission to a journal.

Sequence alignment and phylogeny inference. We assembled and cleaned raw sequences with Sequencher v 5.3 (Gene Codes, Ann Arbor, MI, USA) and aligned the cleaned sequences using the Muscle (Edgar 2004) algorithm in MEGA (v6.0; Tamura *et al.* 2013). For initial phylogenetic exploration, we used maximum likelihood (ML) only, but for the final phylogeny inferences, we used both maximum likelihood (ML) and Bayesian inference (BI) methods implemented in RAxML v8.2.12 (Drummond & Rambaut 2007) and BEAST v2.6.1 (Suchard & Rambaut 2009) software respectively on the CIPRES gateway server (Miller *et al.* 2010). We performed ML analysis employing a rapid bootstrapping algorithm using the program RAxML-HPC BlackBox (v8.2.10; Stamatakis 2014) and considered nodes to be strongly supported when having bootstrap value were above 70% (Hillis & Bull 1993).

To infer Bayesian phylogenies, five independent runs with a chain length of 250 million generations with a log and tree frequency of 10000 generations were performed. A relaxed lognormal clock for mitochondrial genes and a random local clock for nuclear genes. A Yule tree prior and site models selected using the bModelTest plugin (Bouckaert & Drummond 2017). Tracer v1.7.1 (Rambaut *et al.* 2018) was used to visualize and compare convergence and stationarity of each of these individual runs. We then combined them using LogCombiner v.10.4 and annotated our trees using TreeAnnotator v1.10.4 (Drummond & Rambaut 2007) by removing 25% of initial samples as burn-in. We considered nodal support with PP values ≥ 0.95 as significant (Huelsenbeck & Rannala 2004; Mulcahy *et al.* 2011). We implemented our BEAST analysis and post-processing of results through the CIPRES portal (Miller *et al.* 2010). Finally, we used FigTree (v1.4.3; Rambaut 2012) for graphical visualization of the resulting ML and Bayesian trees.

Osteology. We scanned four juveniles of each species of *Sigalegalephrynus burnitelongensis* and *S. gayoluesensis*, along with the specimen of the new species at the Shimadzu Center for Environmental Forensics and Material Sciences at UTA, using a Shimadzu inspeXio SMX-100 CT scanner (Shimadzu, Kyoto, Japan) at 65 kV and 40 µA. Reconstructed raw X-ray data was exported as stacks of 1024 X 1024 16-bit TIFF images. We then rotated and cropped each stack in ImageJ (National Institutes of Health, Bethesda, M.D.) and Contrast Limited Adaptive Histogram Equalization was conducted using the ImageJ plugin Enhance Local Contrast—CLAHE (Zuiderveld 1994). The resulting saved TIFF images were segmented using Drishti v2.6.5 (Limaye 2012) and exported as PLYs. The segmented surfaces were rendered in Avizo (Westenberger 2008) to record characteristics from 3-dimensional surfaces. Micro-CT scans used in this project will be uploaded to MorphoSource with the appropriate project name/file identifier.

RESULTS

Phylogenetic analyses. Our initial maximum likelihood (ML) phylogeny using the *16S* fragment shows a monophyletic *Sigalegalephrynus* and recovers three distinct lineages in the Northern Group, with high nodal supports. A lineage from the Gayo Lues highlands lineage—*S. gayoluesensis*, and two lineages from Gunung Burni Telong—*S. burnitelongensis* and a second unnamed lineage (Fig. 1). Surprisingly, the unnamed lineage, from the G. Burni Telong, was recovered as sister to *S. gayoluesensis*, taxon from a distinct highlands region about 80 km away (aerial distance), not with *S. burnitelongensis*, collected approximately 1.6 km away (actual distance) and with a difference of only 200 m in elevation (Fig. 2). We found that the *16S* uncorrected pairwise genetic distance between the new lineage and *S. burnitelongensis* is higher (8.0%) than to *S. gayoluesensis* (6.3%) (Table 1). However, when using the *16S* fragment (595 bp), the uncorrected p-distance was significantly lower (4% and

3% to *S. burnitelongensis* and *S. gayoluesensis*, respectively). Regardless of amount of the sequence analyzed (*16S* complete or fragment), we observed the same topology with high nodal support for each ML gene tree (*12S*, *tRNA^{Val}*, or *16S)* and for the concatenated mitochondrial gene tree. However, the relationships among species with nuclear genes alone remained unresolved.

Our Bayesian and Maximum Likelihood analyses of concatenated mitochondrial (*12S*, *tRNA^{Val}*, and *16S*) and nuclear (*NCX1* and *POMC*) DNA recovered with high nodal support identical topologies for *Sigalegalephrynus*, the monophyly of the genus, and a sister relationship between *S. gayoluesensis* and *S. burnitelongensis*, with the new lineage being basal to these two species (Fig. 3).

Systematics

Sigalegalephrynus sp. nov.

Figures (4A, 5A, 6A, 7A, 8A, 9A)

Holotype. Museum Zoologicum Bogoriense, Amphibian Collection, MZB.Amph.32042 (field number ENS 19504), subadult female from Burni (Volcano) Geureudong, Kecamatan (District) Tiaman Gajah, Kabupaten (Regency) Bener Meriah, Provinsi (Province) Aceh, Indonesia. 4.77055ºN, 96.80623ºE, 1734 m a.s.l (Fig. 2). Collected along trail above main forest park entrance (Pintu Rimba) by Elijah Wostl, Panupong Thammachoti, Ahmed Muammar Khadafi and Ilham Fonna on 8 August 2015 at 23:10h.

Etymology.

Suggested Common Name.

Diagnosis. The new species of *Sigalegalephrynus* can be diagnosed from its congeners by a unique combination of characters: (1) overall adult size unknown (known individual is subadult and 19.51 mm SVL); (2) lacking parotoid glands; (3) tympanum visible, with elevated annulus not encircled by sharply raised spinose tubercles; (4) naris closer to tip of snout than to eye; eye-naris distance 9.4% SVL, naris-snout distance 4.6% SVL; (5) finger tips II–IV truncated, I rounded; (6) toe tips I, II and III rounded, IV and V truncated; (7) rudimentary hand webbing, moderate in feet; (8) dorsum brown, with hourglass marking with base between eyes and constriction above axillae; a pale diamond situated between scapulae and hourglass constriction; (9) medial dorsal dark band forming the base of the hourglass marking present; (10) dark brown mark followed by white mark on upper lip below eye; (11) flanks with wide dark brown stroke bordered anteriorly and posteriorly by cream color; (12) dorsum tuberculate, tubercles mixed spinose and round; (13) venter areolate, pinkish–yellow without maculation; (14) interocular distance 34% head width; (15) nuptial pad in adult males unknown; (16) finger IV tip does not reach distal phalangeal articulation of finger III (when adpressed); (17) inner and outer metacarpal tubercles of about same length.

Description of holotype and variation. Body moderately robust; head longer than wide, HL/HW = 1.13; head length 38% of SVL; head width 33% of SVL; snout length 11% of SVL; *canthus rostralis* concave; loreal area smooth and concave; eye length 11% of SVL; pupil circular; snout with a vertical row of tubercles forming a keel, giving a mucronate shape dorsally, protruding (slightly sloping back towards mouth) in lateral view; tympanum round with distinct annulus; interorbital space flat; cranial crests absent; no teeth in jaws; tongue tip oval-shaped and longer than wide; skin of dorsal surfaces heavily tuberculate; most tubercles large with keratinization; no dorsolateral, paravertebral, or occipital folds; skin on venter smoother, with very small and round tubercles; circumcloacal region golden yellow. Arms

robust, with moderately developed axillary membrane; forearm length 32% of SVL; hand length 25% of SVL; relative length of fingers: I < II < IV < III; fingers II–IV bearing expanded pads, slightly truncated; webbing formula for hand: I(1.5)-(2)II(1.75)-(2.75)III(2.5)-(2.25)IV; skin of forearm with tubercles; finger I with slightly elongate inner metacarpal tubercle, smaller than the outer metacarpal tubercle; fingers I and II each with one basal round subarticular tubercle, poorly developed.

Thigh length 46% of SVL; tibia length 43% of SVL; tarsal length 20% of SVL; foot length 41% of SVL; relative lengths of toes - I< II< III<V< IV; toes bearing large pads; feet with moderate webbing (Fig. 5), webbing formula for the feet: I(0)–(1.5)II(0)–(2)III(1.5)–(3)IV(3)–(2)V; heels without tubercles; inner metatarsal tubercle moderately developed and elongate; outer metatarsal tubercle distinct; one moderate subarticular tubercle present at the base of first phalanx on each toe; toes with pink toe pads.

Measurements (in mm) of Holotype. SVL 19.51; HL 6.67; HW 6.45; SNL 2.7; ICD 4.20; IND 1.60; END 1.7; NSD 0.90; IOD 2.20; UELW 2.10; TML 1.2; TED 0.5; EW 2.3; IND 1.6; FAL 5.00; HAL 5.70; THL 8.50; TBL 7.85; TRL 4.12; FTL 7.35; IMCL 0.90; IMCW 0.60; OMCL 0.90; OMCW 0.80; IMTL 1.40; IMTW 0.90; OMTL 1.10; OMTW 0.06; F1L 1.30; F2L 2.00; F3L 2.59; F4L 2.60; T1L 1.30; T2L 1.6; T3L 2.40; T4L 4.00; T5L 3.00; F3PD 1.0; F3PB 0.65.

Color of holotype in life. Juvenile holotype (Fig. 4A): dorsum predominantly light brown, distinct inverted triangular marking on the head ends with a whitish-brown diamond marking at the tip; flanks black, with oblique stripes with a white line at the bottom; infraorbital part of maxilla with a dark-brown marking; lore light brown; anterior end of the orbit and naris with light black stripe; dorsum of limbs with distinctive dark crossbar markings; large white tubercles at posterior mandibular articulation below the tympanum;

abdominal surface pinkish, with light brown, lacking maculation; gular region, clavicular, and ventral surface of limbs pink; tips of fingers and toes pink; iris golden yellow, with heavy black reticulations.

Osteology. We compared the skeletons of the northern clade *Sigalegalephrynus* species, the three species from Aceh province. Because *S.* sp. nov. is represented by a subadult and a juvenile female, we scanned four juveniles of similar sizes of *S. burnitelongensis* and four juveniles of *S. gayoluesensis.* We did not find much intraspecific variation among the scans, thus we only present one scan for each of the three species, all females (Figures 8 and 9). As the specimens were juveniles many cartilaginous elements have not yet ossified partially or completely. However, we found the skull of *S.* sp. nov. to be wider than that of the two closely related congeners (Fig. 8). The width of the terminal phalanx of each finger appeared to be much wider in *S.* sp. nov. than in *S. burnitelongensis* and *S. gayoluesensis* (Figures 9). The lateral ridges at the anterior of the urostyle seem less prominent in *S.* sp. nov. than in *S. burnitelongensis* and *S. gayoluesensis* (Fig. 11).

Color of holotype in preservative. Differing slightly from that in life, pinkish coloration turned brown, and the venter has turned whitish-grey (Figure 5A, 6A, 7A).

Distribution and natural history. New *Sigalegalephrynus* species is known only from the forest of Burni (Volcano) Telong, 1734 m a.s.l. (Fig. 2), in the twin-volcano complex of Burni Telong, just north of Takengong, central Aceh, Sumatra. Another species, *S. burnitelongensis* also occurs on the same volcanic complex, at the slopes of Burni Telong, the second volcano in the complex. The type locality of *S. burnitelongensis* is separated from the type locality of new *Sigalegalephrynus* species by only about one kilometer and 200 m in elevation, on the western flank of the volcanic complex. Likely, the two species occur at least in narrow sympatry, with new *Sigalegalephrynus* species probably having most of its

distribution higher in the mountain. At least four *Sigalegalephrynus* species have been found in close affinity with streams. In fact, *S. burnitelongensis* and new *Sigalegalephrynus* species were found along the banks of two streams flowing downhill and side by side. The single known individual of new *Sigalegalephrynus* species was found sitting on a small leaf of a shrub 1.5 m above the ground. The post-euthanization weight of the animal was 0.47 g.

Comparisons. The new *Sigalegalephrynus* species can be easily distinguished from *S. burnitelongensis* by its' prominent and large diamond marking on the dorsum (absent or not prominent in *S. burnitelongensis*), blackish and dark-brown flanks roughly demarked with a brownish line extending from the suborbital region to the end of the inguinal joint on the bottom and from the abdominal region the inguinal region on the top (completely demarked with white line in *S. burnitelongensis*), and by the presence an well-defined inverted triangle marking on the dorsum originating in the interocular region that joins with a diamond-shaped marking in the inter-scapular region (absent or faint in *S. burnitelongensis*). It differs from *S. gayoluesensis* by having an unmarked (not maculated) ventrum. The new *Sigalegalephrynus* species differs from *S. burnitelonensis*, *S. mandailinguensis*, *S. minangkabauensis* and *S. harveyi* by its fourth finger going beyond the distal phalangeal articulation of finger III (vs. touching the terminal (distal) phalangeal articulation of Finger III in *S. mandailinguensis S. minankabauensis*, and not going beyond the articulation in *S. burnitelonensis* and *S. harveyi*), having prominent diamond-shaped marking on the dorsum (lacking in *S. mandailinguensis S. minankabauensis* and *S. harveyi* and adult *S. burnitelonensis*).

DISCUSSION

The niche modeling results presented by Sarker *et al.* (2019) indicate that many new species of *Sigalegalephrynus* await discovery on Sumatra. However, their niche modeling was based

on the non-sympatric occurrence of Puppet Toad species in Sumatra. The discovery of this sympatric new species ascertains and bolsters their niche modeling results. With this discovery, *Sigalegalephrynus* is again confirmed as the most diverse species-containing endemic genus of amphibian in Indonesia. Subgneric sympatry in Southeast Asian bufonid toads is not uncommon, for example, between *Leptophryne borbonica* and *L. cruentata* (Kusrini *et al.* 2016), *Pelophryne penrissenensis* and *P. guentheri* (Matsui *et al.* 2017), *Phrynoidis asper* and *P. juxtasper* (Inger 1964), and *Ansonia platysoma* and *A. kanak* (Matsui *et al.* 2020).

Sigalegalephrynus is one of the four most mysterious and micro-endemic bufonid genera in South and Southeast Asia (other are: *Pseudobufo*—occurs in Sumatra and Borneo, *Ghatophryne*—occurs in Western Ghats of India, and *Parapelophryne*—occurs in Hainan Island of China), and has been very important in understanding the evolutionary relationships of bufonid frogs of South and Southeast Asia (Chan & Grismer 2019). Lack of adequate molecular data from these genera, along with many other factors, such as, lack of informative sites per genes and a rapid radiation is often to blame for the poor understanding of toad phylogenetic relationships (Whitfield & Lockhart 2007, Whitfield & Kjer 2008, Smart *et al.* 2017, Chan & Grismer 2019). By adding more molecular data (mtDNA and nuDNA), from all species of *Sigalegalephrynus*, we provide a better multilocus framework for this basal Asian genus to improve future taxonomic and phylogenetic studies.

Our study revealed that using just one small fragment of a gene could underestimate genetic divergence and hence species diversity. When we analyzed 595 bp of the *16S* gene, we found a genetic divergence between the new *Sigalegalephrynus* species and *S. gayoluesensis* and *S. burnitelongensis* of only 3% and 4% respectively. However, genetic divergence rose to 6.8% and 8.4%, respectively, when we sequenced the complete *16S* gene,

1395 bp long. This finding signifies the importance of having more molecular data in phylogenetic studies.

For our study, we not only used mtDNA data but also nuDNA data. Mito-nuclear discordance in phylogenetics is an important issue in identifying toad species (Gonnçalves *et al*. 2007, Fontenot *et al.* 2011, Thomé *et al.* 2012). We observed some mito-nuclear discordance, recovering different relationships when adding nuclear molecular data. Our concatenated tree recovered *S.* sp. nov. as basal other two species from Aceh (Fig.2); otherwise, it showed a sister taxon relationship with *S. gayoluesensis* based on only mitochondrial molecular data (Fig. 1). From the biogeographical point of view, the concatenated phylogeny is more plausible than the mitochondrial tree only. More divergence between the new *Sigalegalephrynus* species and *S. burnitelongensis* happened for most probably Burni Telong has been colonized at least twice. The first colonization event occurred by new *Sigalegalephrynus* species followed by a second colonization event by *S. burnitelongensis* when *S. burnitelongensis* separated from their common ancestor with *S. gayoluesensis.* The discordance between mitochondrial and nuclear trees highlights using multiple loci from both mitochondrial and nuclear DNA is crucial for understanding the phylogeny of taxa, and if possible, we should consider genome data from next-generation sequencing for future studies.

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APPENDIX I.

Additional Specimens Examined.

Sigalegalephrynus burnitelongensis (36).—INDONESIA: Aceh: Kambupaten Bener Meriah: MZB.Amph.30413—Holotype (adult male); UTA A-65788 and UTA A-6549—Paratypes; UTA A−65493−509 (17); and MZB.Amph.26016−031 (16) 4.76455ºN, 96.80138ºE, 1519 m a.s.l.

Sigalegalephrynus gayoluesensis (12).—INDONESIA: Aceh: Kambupaten Gayo Lues: MZB.Amph.30411—Holotype (adult male), 4.22588ºN, 97.18915ºE, 1850 m. a.s.l.; UTA A-65490, MZB.Amph.26035, MZB.Amph.26037—Paratypes, 4.22580ºN, 97.1886º1E, 1844 m. a.s.l.; UTA A-65488−489, 65789 (subadult and two juveniles, respectively, 1827 m. a.s.l., 4.2239ºN, 97.18718ºE); 65790 (subadult, 1826 m. a.s.l., 4.22487ºN, 97.18769ºE); and MZB.Amph.26032 (juvenile, 1827 m. a.s.l., 4.22357ºN, 97.186551º E); 26033 (juvenile,1827 m. a.s.l., 4.2239ºN, 97.18718ºE); 26034, 26036 (two juveniles,1826 m. a.s.l., 4.22487ºN, 97.18769ºE).

Sigalegalephrynus harveyi (2).—INDONESIA: Sumatera Selatan: Kambupaten Pagar Alam: Gunung Dempo, 1826 m. a.s.l., 4.040980ºS, 103.14810ºE, MZB.Amph.30412—Holotype (adult male); 1878 m. a.s.l., 4.03923ºS, 103.14730ºE, UTA A-65474—Paratype.

Sigalegalephrynus mandailinguensis (4).—INDONESIA: Sumatera Utara: Kabupaten Mandailing Natal: Gunung Sorikmarapi (West side), 1383 m. a.s.l., 0.701648ºN, 99.552628ºE, MZB.Amph.25736—Holotype (adult male); trail between the Tano Bato to Sapo Tinjak road and Lake Saba Begu, Batang Gadis National Park, 1297 m. a.s.l., 0.708668ºN, 99.519538ºE, MZB.Amph.25737—Paratype (male); 1299 m. a.s.l., 0.708668ºN, 99.519538ºE, UTA A-63562—Paratype (male), UTA A-63561—Paratype (male).

Sigalegalephrynus minangkabauensis (1).—INDONESIA: Jambi: Kabupaten Kerinci: Gunung Kunyit, 1428 m. a.s.l., 2.260138ºS, 101.495128ºE, MZB.Amph.25738—Holotype.

Table 1. Uncorrected p-distance in 12S rRNA (956 bp—below diagonal) and 16S rRNA (1411 bp—above diagonal) sequences of toad species

	1	2	3	4	5	6	7	8	9	10	11	12
1. *Ingerophrynus boporcatus*		0.161	0.169	0.173	0.150	0.150	0.171	0.164	0.176	0.158	0.159	0.169
2. *Leptophryne borbonica*	0.098		0.158	0.174	0.180	0.180	0.168	0.166	0.179	0.156	0.171	0.155
3. *Pelophyne sp.*	0.122	0.142		0.186	0.201	0.201	0.170	0.166	0.169	0.159	0.171	0.164
4. *Phrynoidis asper*	0.089	0.099	0.145		0.169	0.169	0.159	0.154	0.165	0.143	0.151	0.136
5. *Pseudobufo subsubasper*	0.089	0.100	0.146	0.090		0.000	0.180	0.182	0.185	0.175	0.175	0.175
6. *Pseudobufo subsubasper*	0.086	0.097	0.143	0.087	0.002		0.180	0.182	0.185	0.175	0.175	0.175
7. *Sigalegalephrynus* sp. nov.	0.105	0.113	0.152	0.109	0.118	0.115		0.080	0.063	0.072	0.129	0.096
8. *S. burnitelongensis*	0.102	0.111	0.147	0.097	0.122	0.119	0.052		0.065	0.100	0.118	0.098
9. *S. gayoluesensis*	0.103	0.118	0.155	0.108	0.117	0.114	0.037	0.066		0.092	0.125	0.110
10. *S. harveyi*	0.071	0.097	0.128	0.085	0.097	0.094	0.083	0.089	0.092		0.109	0.072
11. *S. mandaiminguensis*	0.070	0.099	0.131	0.091	0.094	0.091	0.080	0.089	0.083	0.036		0.106
12. *S. minangkabaueneis*	0.084	0.115	0.134	0.115	0.106	0.104	0.097	0.102	0.102	0.047	0.048	

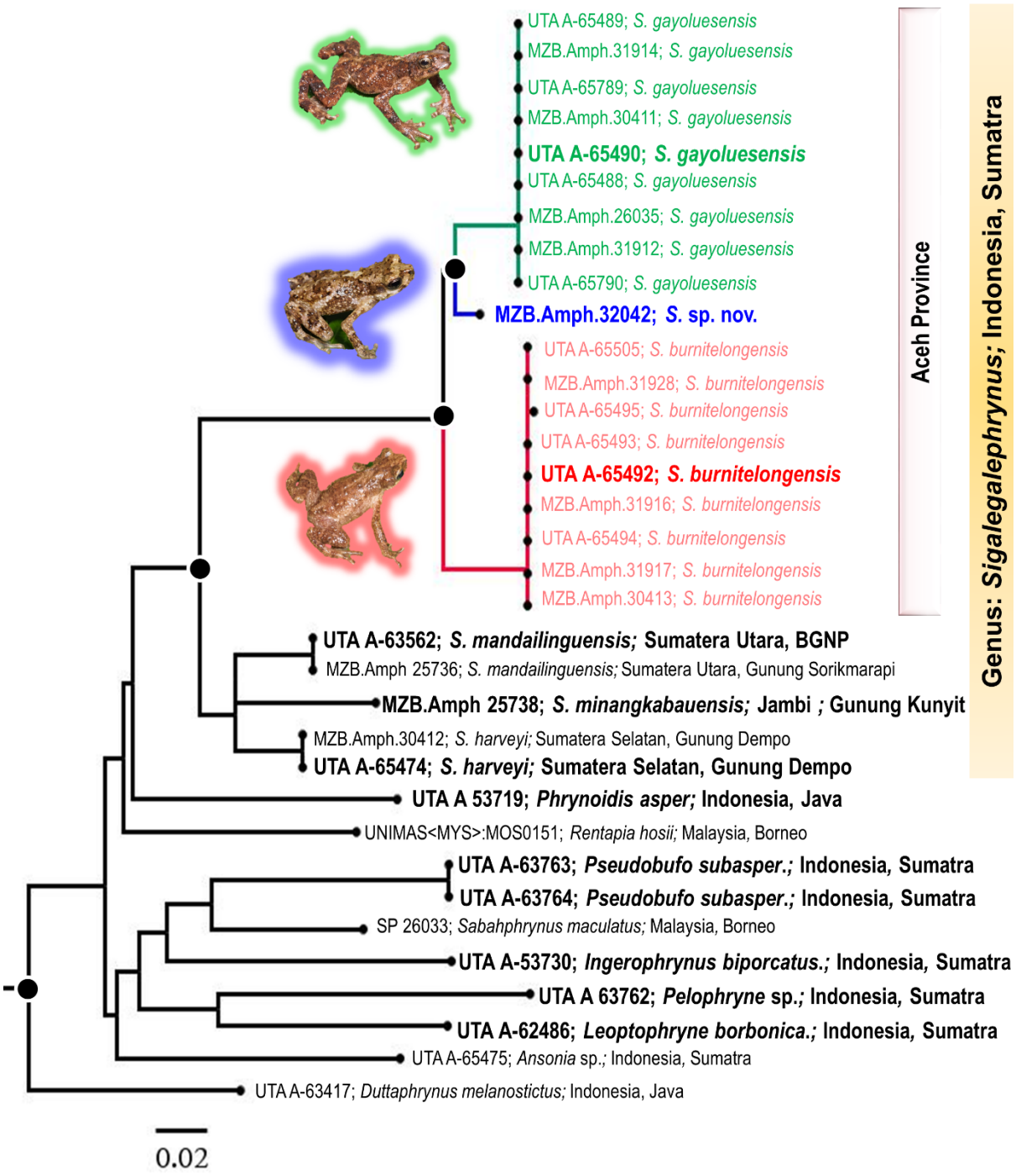

Figure 1. Estimated phylogeny of Sigalegalephrynus based on a fragment of 16S mitochondrial rRNA, depicted as a maximum-likelihood tree with bufonid outgroups. Non-bufonid and New World bufonid ourgroup taxa have been trimmed out off the tree. Internal nodes with large black circles indicate having significant support. OTUs in bold indicates these are specimens used for further mitochondrial and nuclear sequencing.

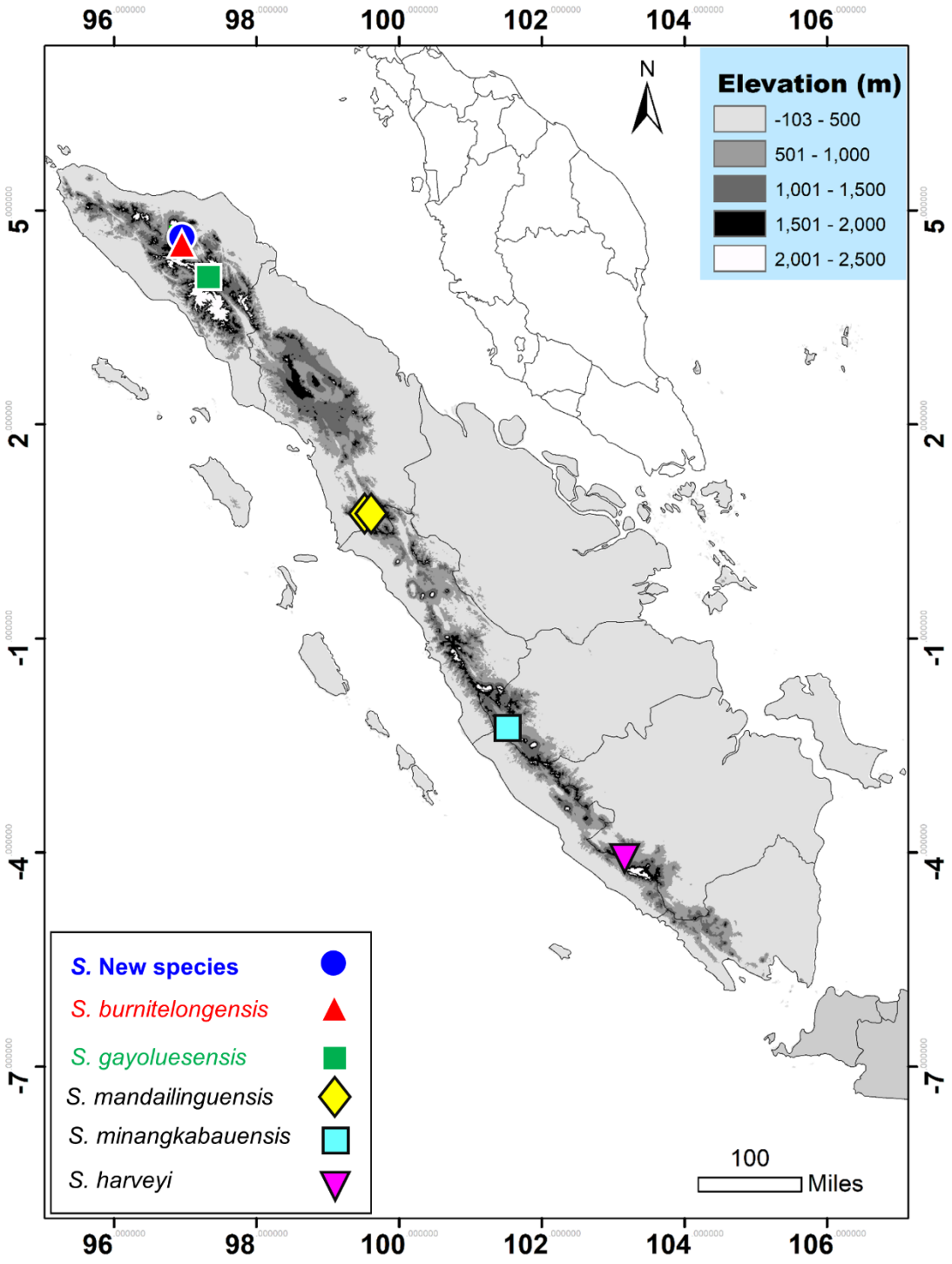

Figure 2. Map of Sumatra showing the known distribution of *Sigalegalephrynus* species

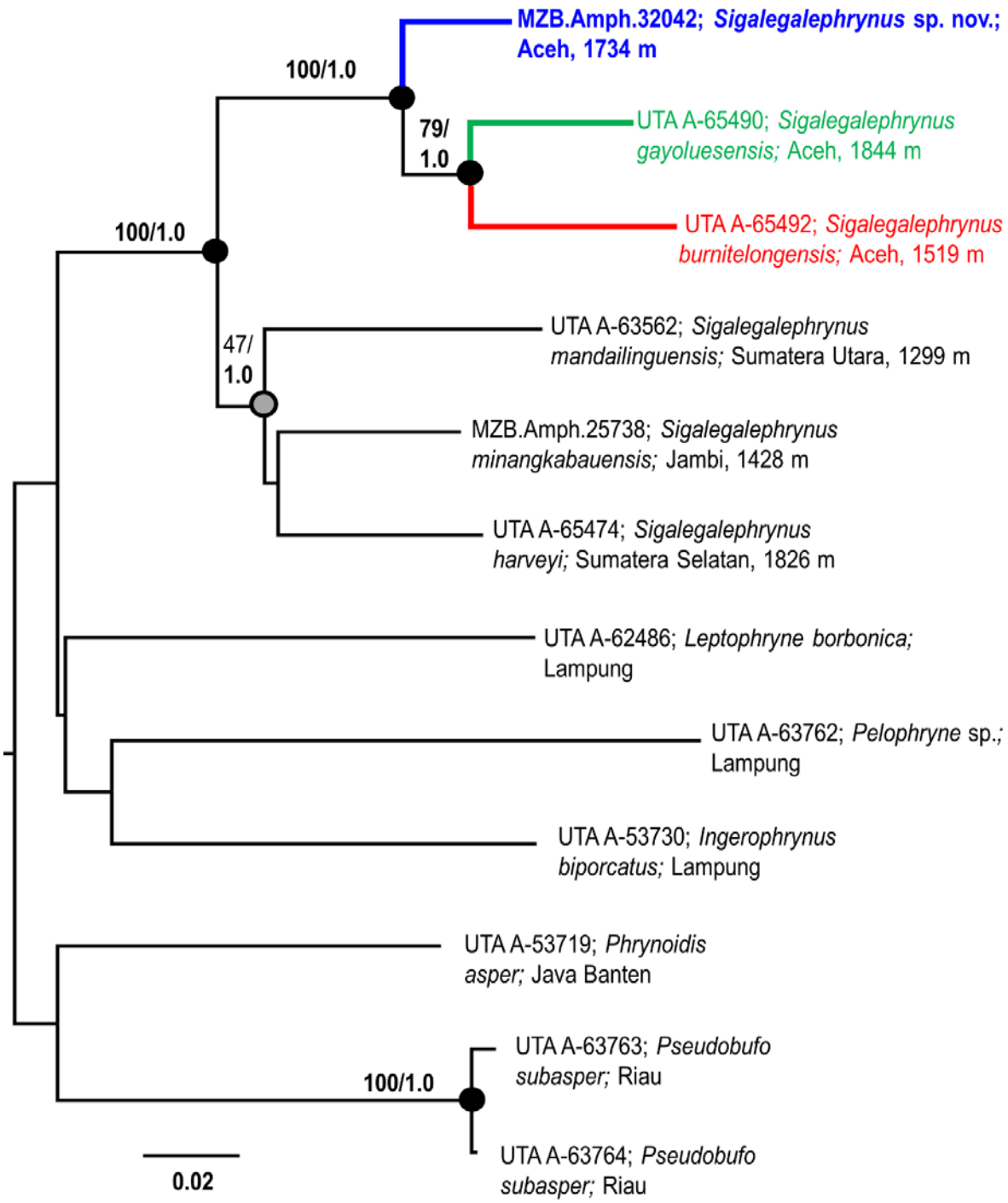

Figure 3. Estimated phylogeny of Sigalegalephrynus based on mitochondrial rRNA (12S, 16S and tRNAVal) and nuclear (POMC and NCX1) dataset, depicted as a maximum-likelihood tree with bufonid outgroups. The non-bufonid outgroup *Dryophytes arenicolor* is not shown.

Figure 4. Dorsal, dorsolateral and ventral aspects of A) *S.* sp. nov. (MZB.Amph.32042—holotype, subadult female), B) *S. burnitelongensis*— UTA A.-65493 (subadult female), and C) *S. gayoluesensis* MZB.Amph.26037 (adult female) in life.

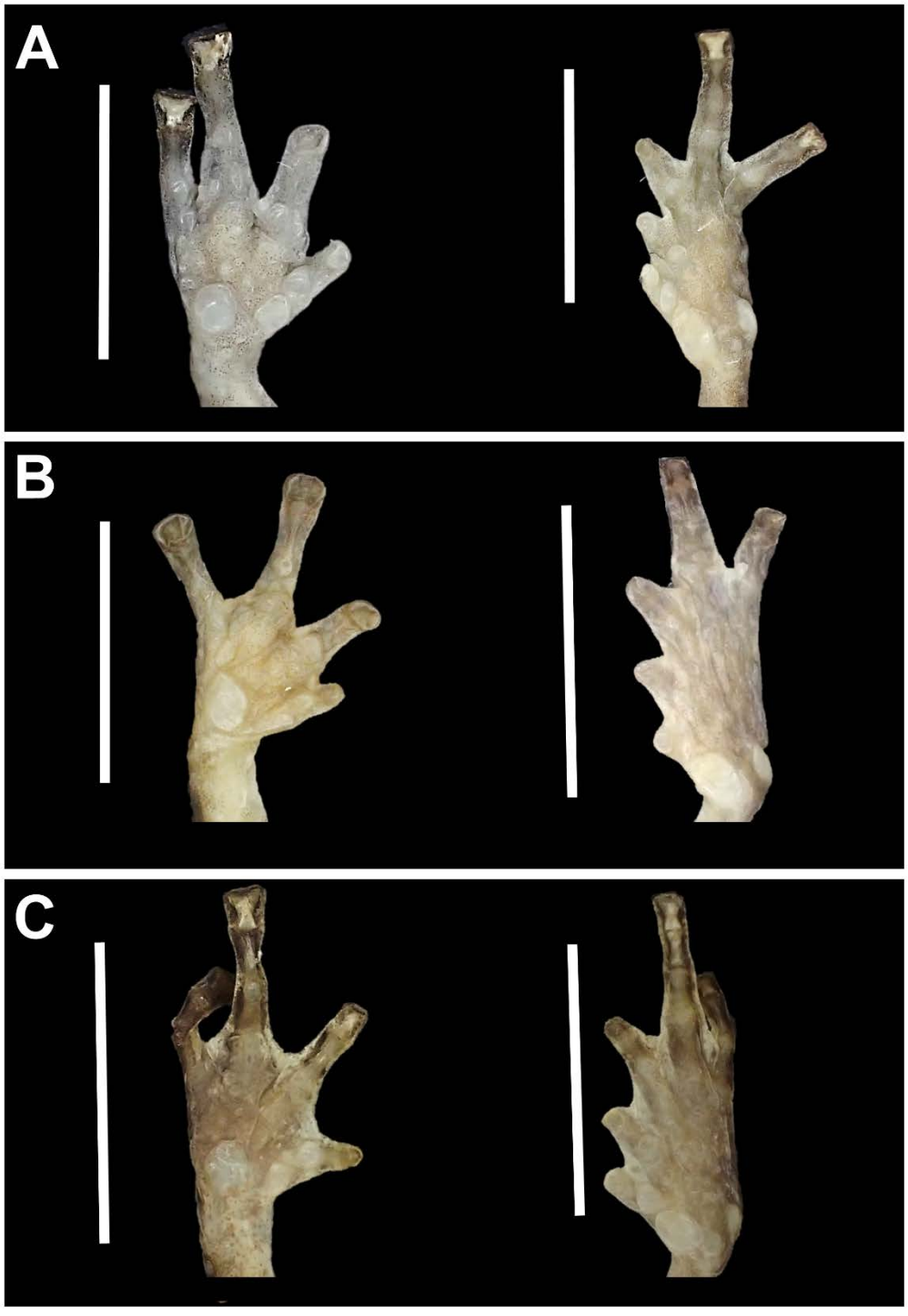

Figure 5. Palmar and plantar surfaces juveniles of A) *S.* sp. nov. (MZB.Amph.32042—Holotype), B) *S. burnitelongensis* (UTA A-65493) and C) *S. gayoluesensis* (UTA A-65489). Scale bar = 5 mm.

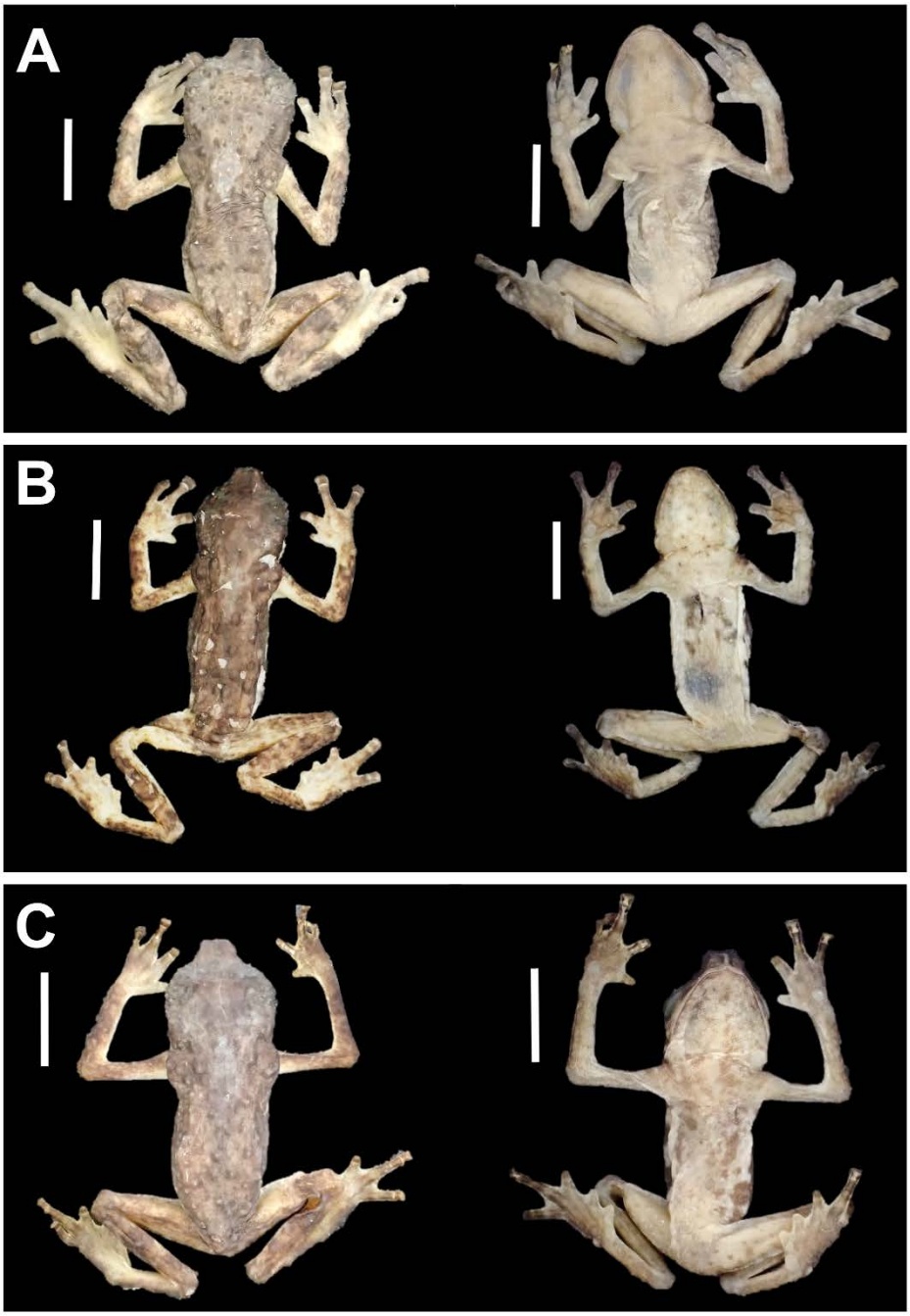

Figure 6. Dorsal and ventral aspects of juveniles of A) *S.* sp. nov. (MZB.Amph.32042—Holotype), B) *S. burnitelongensis* (UTA A-65493) and C) *S. gayoluesensis* (UTA A-65489) in preservation. Scale bar = 5 mm.

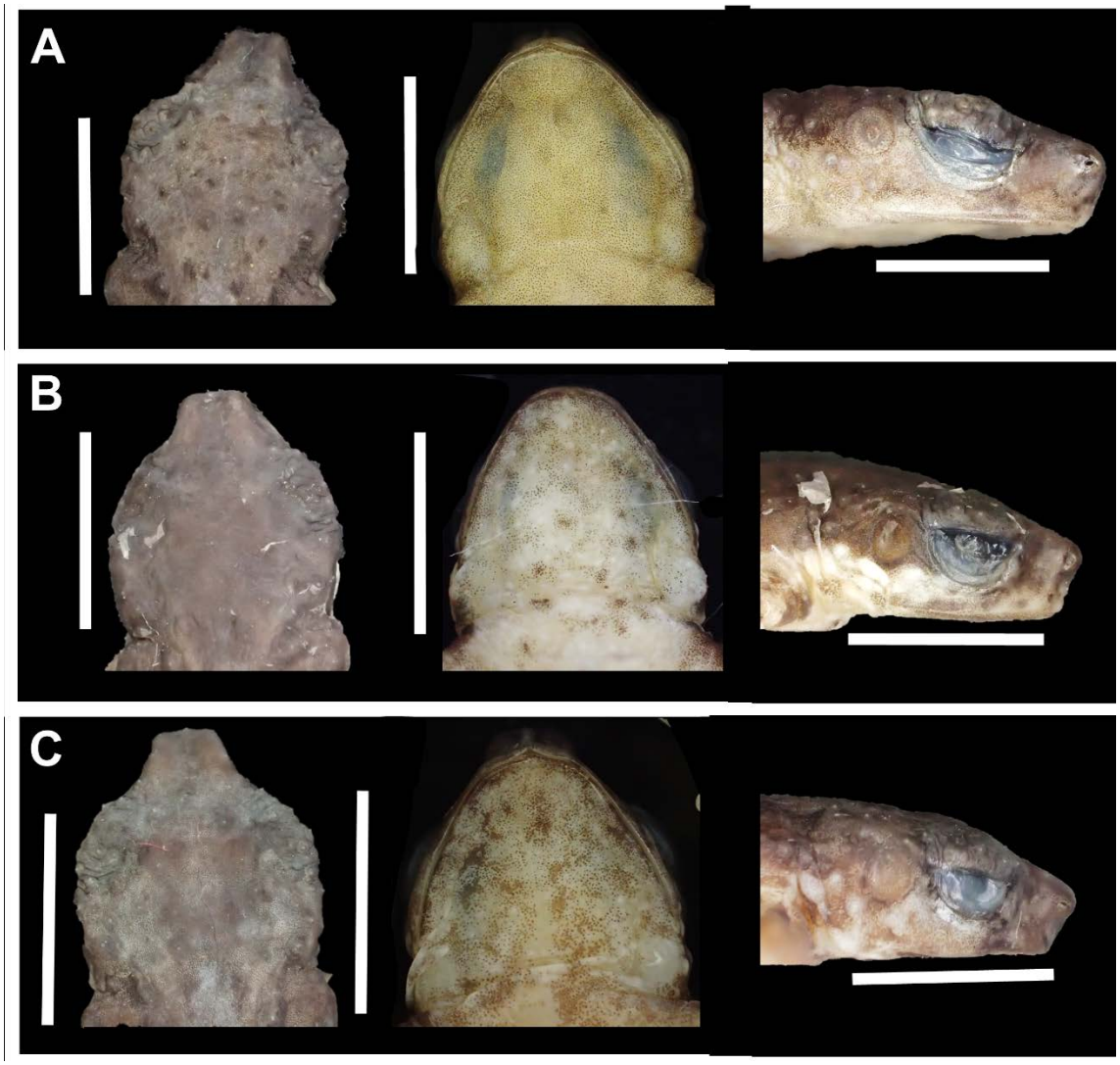

Figure 7. Head, gular region, and profile view of juveniles of A) *S.* sp. nov. (MZB.Amph.32042—Holotype), B) *S. burnitelongensis* (UTA A-65493) and C) *S. gayoluesensis* (UTA A-65489) in preservation. Scale bar = 5 mm.

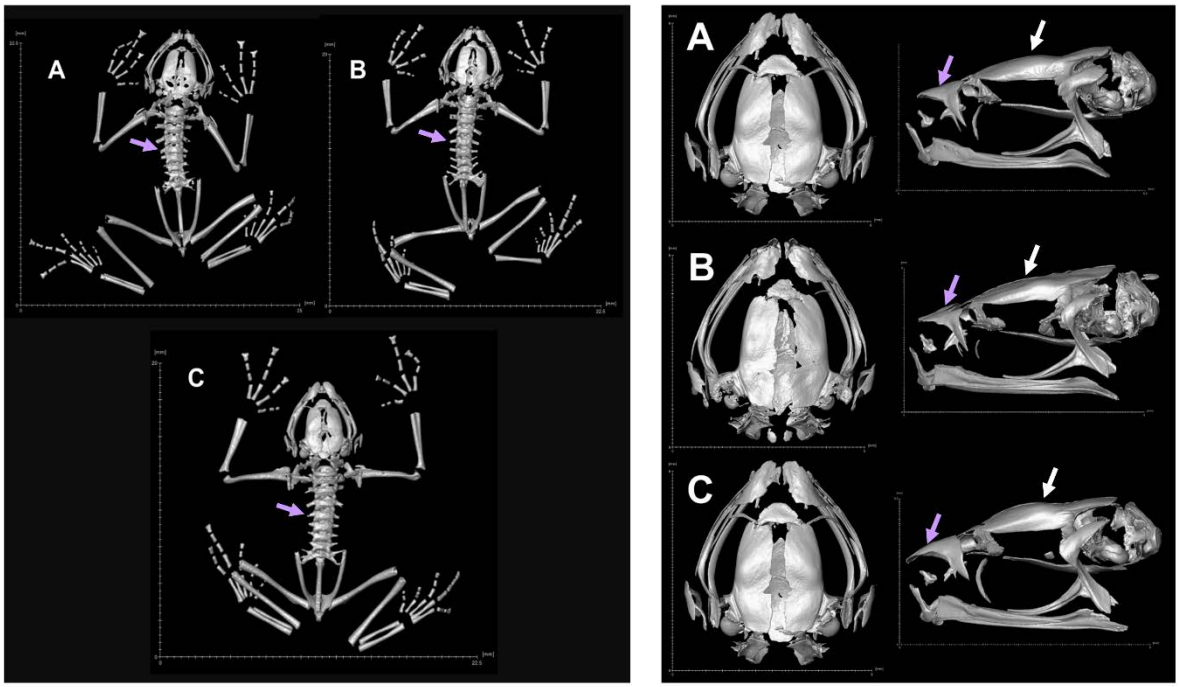

Figure 8. Left: Dorsal view of micro-CT scanning of entire skeleton of A) holotype *S.* sp. nov. (MZB.Amph.32042—Holotype), B) *S. burnitelongensis* (UTA A-65493), and C) *S. gayoluesensis* (UTA A-65489) (Arrows highlight the relative length of the transverse processes to the body of the vertebrae). Right: Dorsal and lateral view of micro-CT scanning of skull of A) holotype of *S.* sp. nov. (MZB.Amph.32042—Holotype), B) *S. burnitelongensis* (UTA A-65493), and C) *S. gayoluesensis* (UTA A-65489) (Arrows highlight the differences in shape of the nasal bone and curvature of the frontoparietal bone).

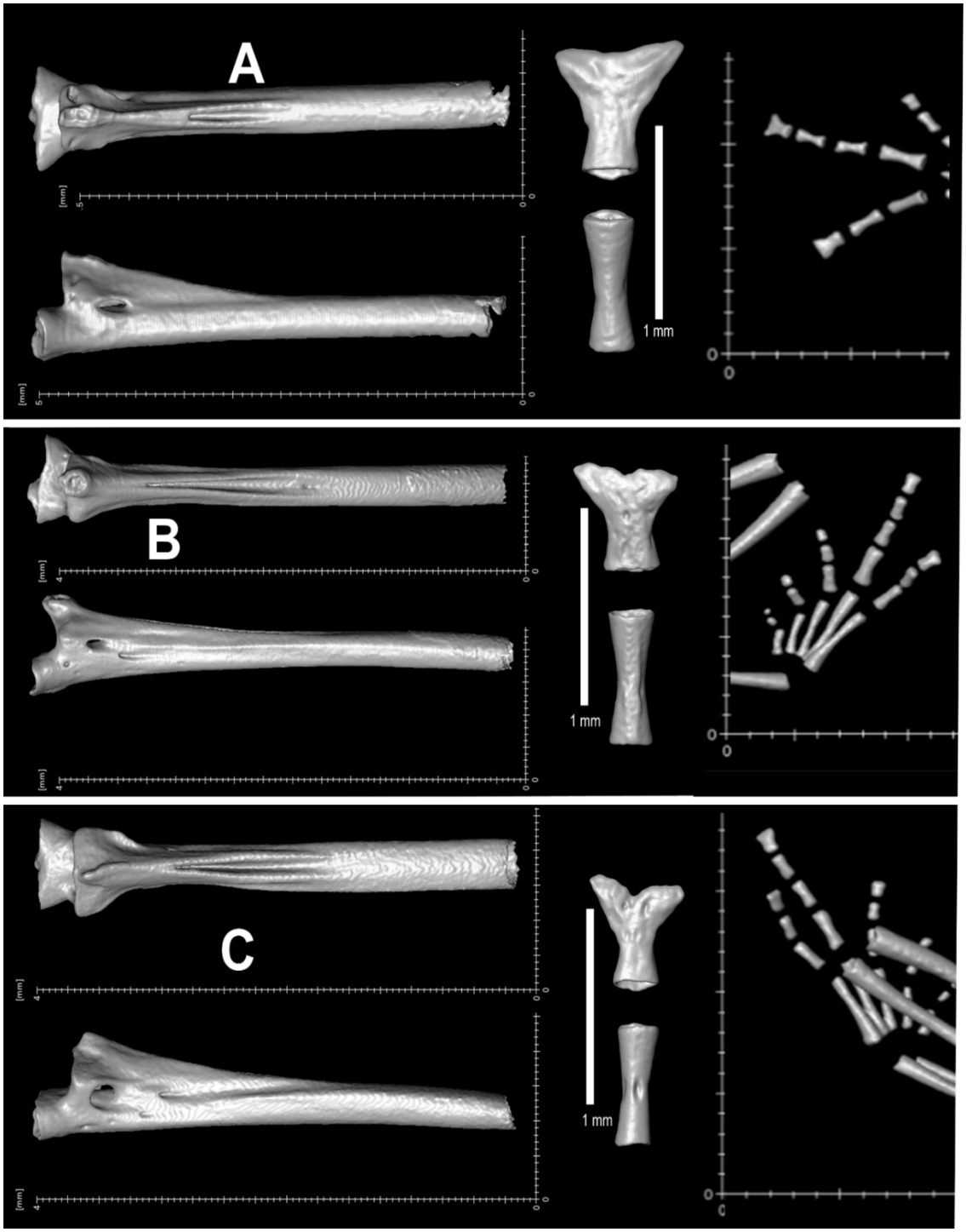

Figure 9. Left: Dorsal (top), lateral (bottom) view of micro-CT scanning of urostyle. Middle: last two digits of finger IV. Right: micro-CT scanning of last phalanges of toes IV and V of A) holotype of *S.* sp. nov. (MZB.Amph.32042—Holotype), B) *S. burnitelongensis* (UTA A-65493), and C) *S. gayoluesensis* (UTA A-65489).

CHAPTER FOUR

Multilocus phylogeny of the Southeast Asian giant river toads (Bufonidae: *Phrynoidis*) reveals an endemic new species from Sumatra emphasizing conservation challenges

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ABSTRACT

Although sequences from few representative *Phrynoidis* specimens have *occasionally* been used in molecular studies of higher-level amphibian systematics, so far no studies have been conducted on the molecular divergences and genetic structure of different species and populations of this genus. Also, comprehensive morphological studies on this genus are very scant. In this study, we used two mitochondrial and four nuclear gene sequences to assess phylogenetic and systematic relationships of different populations of the genus. We used 107 adult individuals (43 females and 64 males) for our morphometric analyses. We identified three divergent, distinct, and independently evolving lineages in the genus. Our study reveals that one new lineage, which inhabits the highlands of Sumatra, wes previously misidentified as *P. juxtasper.* This new lineage shows substantial genetic divergence from *P. asper* and from *P. juxtasper*, which is comparable to the amount of genetic divergence between *P. asper* and *P. juxtasper*. The new lineage of Sumatra also demonstrates morphological differentiation from the other two currently recognized species. This lineage is completely allopatrically separated from *P. juxtasper* endemic to Borneo, additionally, the current distribution shows populations of the new lineage inhabits in high-elevation habitats in comparison to the lowland dwelling *P. asper* of Sumatra. Hence, we consider the new lineage and highland *Phrynoidis* population of Sumatra as a strong candidate for a new species.

Key Words: Anura, Conservation, *Phrynoidis asper*, *Phrynoidis juxtasper*, *Phrynoidis* species nov., Southeast Asia, Sunda-shelf

INTRODUCTION

Gravenhorst (1829) first described a bufonid species as *Bufo asper* which was transferred to a monotypic new genus *Phrynoidis* by Fitzinger (1843). Fitzinger also published an illustration

of *Bufo asper* in 1842—see Fitzinger in Treitschke (1842)—although Boulenger (1882) considered *Phrynoidis* is a junior synonym of the genus *Bufo*. Inger (1964) accepted the synonymy by Boulenger and described the second species of the genus as *Bufo (=Phrynoidis*) *juxtasper* based on morphological studies. However, in the comprehensive study of molecular taxonomy and systematics of amphibians of the world, Frost *et al.* (2006) resurrected the genus *Phrynoidis* and removed it from synonymy with the genus *Bufo*.

Both the *Phrynoidis* species have specific ecological requirements. They breed in lotic water, from where the common English name of the genus—river toad came from, to breed (Inger *et al.* 2017). The type locality of *P. asper* and *P. juxtasper* is West Java of Indonesia and Sabah Province of Bornean Malaysia respectively. While *P. asper* has an extensive distribution that ranges from southern Myanmar, Thailand, and Vietnam to East Java along with Borneo and Sumatra, *P. juxtasper* is restricted to Borneo and Sumatra only (Inger 1964, 1966, Inger *et al.* 2017, Frost *et al.* 2020). Although *P. juxtasper* is present all over Borneo, it is extremely abundant in the lowland of Sabah. In Borneo, both these two species found in sympatry without intergradation (Inger 1964, 1966). However, there is an elevational variation in the distribution of these two species.

Elevational distribution of *Phrynoidis asper* population ranges from near the sea level to 360 m a.s.l. in Myanmar and Thailand (Mulkahy *et al.* 2018, FMNH collection records). In Vietnam, it occurs below 700 m a.s.l. (Frost *et al.* 2020). However, in Borneo, this species can be found from near the sea level to as high as 1600 m a.s.l. (Inger 1966, Das 2006, Das *et al.* 2007, Grafe and Keller 2009, Afif *et al.* 2012, Sheridan *et al.* 2012, Inger *et al.* 2017, Hass *et al.* 2018, Amram *et al*. 2018, Frost *et al*. 2020). On the other hand, *P. juxtasper* population is very abundant in the lowlands of Borneo and can also found at 1000 m a.s.l. (Inger *et al.* 2017). Distribution records of *Phrynoidis* populations in Sumatra in published articles are not

abundant, moreover, no studies have focused on the distribution pattern of *Phrynoidis* populations in Sumatra so far.

Several studies used molecular data of *Phrynoidis* in their evolutionary studies (Pramuk 2006, Matsui *et al.* 2007, van Bocxlaer *et al*. 2009, Pyron and Weins 2011, Portik and Pappenfus 2015, Chandramouli *et al.* 2016, Smart *et al.* 2017, Haas *et al.* 2018, Mulcahy *et al.* 2018, Chan and Grismer 2019, Sarker *et al.* 2019). These data have shown that there has been shallow genetic structure in populations of *P. asper* across mainland Southeast Asia, Borneo, Sumatra, and Java. However, none of these studies included molecular data from Sumatran *Phrynoidis* populations. On the other hand, Inger (1964) has done the only comprehensive morphological study on this genus using. In his mensural studies, he used *P. asper* and *P. juxtasper* specimens collected mainly from Borneo to recognize *P. juxtasper* as the new species. He also examined few male specimens from Thailand, Sumatra, and Peninsular Malaysia and mentioned differences in nuptial pads and vocal sacs. In 1966, published a book on Bornean amphibians and mentioned differences of parotoid glands length and width ratio between *P. asper* and of *P. juxtasper* and refrained from elaborating on morphological differences of Sumatran *Phrynoidis* populations.

In this study, we aimed to bridge the gap of information of the genus and examined both morphological and molecular data. We assessed the taxonomic and evolutionary relationship of the Sumatran *Phrynoidis* population using an integrative approach, including morphological, molecular, and ecological data (de Quiroz 2007). This is the first study that examines both molecular and morphological data and evaluates the evolutionary relationship among the populations of the species. Based on the morphological and molecular differences, we herein describe a new *Phrynoidis* from Sumatra.

MATERIALS AND METHODS

Taxon sampling and DNA sequencing. Specimens used in this study were collected alive from Indonesia from December 2013 to November 2016. Along with the weather and habitat conditions, we recorded the latitude, longitude, and elevation using a handheld global positioning system device (Garmin® GPSMAP 64s) with the datum set to WGS84 at the time of collection of specimens. We fixed our specimens in 10% formalin before transferring to 70% alcohol. Before fixation, we took liver or muscle tissue samples and preserved them in n 1.5 mL of cell lysis buffer solution (0.5 M Tris/0.25% EDTA/2.5% SDS, pH = 8.2). Immediately after euthanization, we also took photographs of live animals along with pictures of the dorsal, ventral, and lateral aspects. We strictly followed Institutional Animal Care and Use Committees (IACUC; number UTA IACUC A12.004) protocols from collecting to preserving all our specimens. Finally, we deposited all specimens at the Museum Zoologicum Bogoriense (MZB), and the Amphibian and Reptile Diversity Research Center (ARDRC) at the University of Texas, Arlington (UTA). All other museum acronyms follow Sabaj Perez (2014).

Initially, for barcoding purposes, we sequenced one fragment of the 16S mitochondrial gene (~530 bp) from 33 new *Phrynoidis* specimens. We then examined pairwise genetic divergence and phylogenetic relationships by incorporating them with all of the *Phrynoidis* 16S sequences available on the GenBank database. We also used at least one sequence from each of the extant genera of the region to test the monophyly of the *Phrynoidis* sequences. After an initial examination of the newly generated sequences, we used ten primer combinations and amplified up to 2540bp of mitochondrial 12S and 16S genes along with the flanking $tRNA^{val}$ gene (Goebel *et al.* 1999, Feller & Hedges 1998, Titus & Larson 1996, Hedges 1994, Palumbi *et al.* 1991, Wilkinson *et al.* 1996). We also sequenced four nuclear genes following different sequencing protocols (NCX1—Bossuyt & Milinkovich 2000,

CXCR4—Biju & Bossuyt 2003, RAG1—Hoegg *et al.* 2004, and POMC—Wiens *et al.* 2005, Pramuk 2006) from 8 specimens. We visually assessed PCR success on a 1% agarose gel, following PCR product purification with Serapure beads.

Sequencing reactions with an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) were performed at the Genomic Core Facility (GCF) of the Department of Biology at the University of Texas at Arlington. Before sequencing, for isolating DNA and purifying PCR products, we used Serapure beads following the Agencourt protocol (Beckman Coulter Co., Fort Collins, CO, USA) after Rohland & Reich (2012).

Sequence alignment and phylogenetic analyses. Sequence alignment was done using Sequencer v5.3 (Gene Codes, Ann Arbor, MI, USA) to assemble and clean raw sequences. We then used MEGA version X (Kumar *et al*. 2018) to align our sequences implementing the ClustalW algorithm (Larkin *et al.* 2007). CIPRES scientific webserver (Miller *et al.* 2010) were used to run maximum likelihood analysis implemented on RAxML (Drummond and Rambaut, 2007) for exploring initial phylogeny, and we used 65 sequences of one fragment of 16S gene out of which 48 were from *Phrynoidis* species. We included all of the available 15 GenBank haplotype sequences for this analysis and 33 new sequences were generated for this study. To examine monophyly of *Phrynoidis* species, we included sequences from at least one representative bufonid species from all of the extant Southeast Asian bufonid genera as ingroup and one New World hylid frog—*Dryophytes arenicolor* sequence as outgroup.

After initial exploration of phylogeny, we then used full 12S and 16S rRNA mitochondrial sequences along with the flanking $tRNA^{val}$ gene (~2540bp) to analyze the phylogenetic relationships, by running both maximum likelihood— implemented on RaxML— and Bayesian analysis implemented on BEAST2.6.1 (Suchard and Rambaut, 2009), among the *Phrynoidis* lineages by choosing fewer *Phrynoidis* OTUs and outgroups

which have comparable lengths of sequences. Finally, we added four nuclear sequences in our supermatrix and ran both maximum likelihood and Bayesian analyses. Nodes with bootstrap values ≥ 70% and posterior probability values ≥ 0.95 were considered strongly supported. All sequences generated for this project will be submitted to GenBank.

Morphological data and analyses. We adapted our morphological terminology from Sarker *et al.* (2019), which were based on Matsui (1984), Duellman (2001), and Kok and Kalamandeen (2008). We used digital calipers and an ocular micrometer to the nearest 0.1mm, on a stereoscope, for measuring the specimens. We measured: 1) snout–vent length (SVL), tip of the snout to anterior margin of the vent; 2) head length (HL), posterior angle of jaw to tip of snout; 3) head width (HW), measured ventrally at the angles of jaw, excluding warts; 4) snout length (SNL), anterior corner of eye to snout tip; 5) eye-Naris length (ENL); 6) naris to snout length (NSL); 7) eye length (EYL), horizontal distance from anterior to posterior joints of upper and lower eyelids; 8) tympanum width (TMW), horizontal width of tympanum; 9) tympanum to eye distance (TED); 10) parotoid gland length (PGL); 11) parotoid gland width (PGW); 12) internarial distance (IND), distance between anterior ends of nares; 13) interorbital distance (IOD), minimal distance between upper eyelids; 14) intercanthal distance (ICD), distance between anterior edges of canthi; 15) forearm length (FAL), tip of elbow to proximal margin of outer metacarpal tubercle; 16) hand length (HL), proximal marg`in of metacarpal tubercle to Finger III tip; 17) inner metacarpal tubercle length (IMCL), from the anterior to the posterior end of the inner metacarpal tubercle; 18) inner metacarpal tubercle width (IMCW), greatest width of inner metacarpal tubercle measured perpendicularly to IMCL; 19) outer metacarpal tubercle length (OMCL), from the anterior to the posterior end of the outer metacarpal tubercle; 20) outer metacarpal tubercle width (OMCW), greatest width of outer metacarpal tubercle measured perpendicularly to OMCL; 21) thigh length (THL), center of cloaca to distal surface of knee, appressed; 22)

tibia length (TBL), greatest length of tibia when positioning hind limb in a Z pattern; 23) tarsus length (TRL), tibio-tarsal articulation to proximal margin of outer metatarsal tubercle; 24) foot length (FTL), proximal margin of outer metatarsal tubercle to Toe IV tip. All morphological data were collected by a single observer (TRL).

To test the morphological distinctiveness of each group, we conducted linear discriminant analysis (LDA) in PAST 3.24 (Hammer *et al.* 2001). We first did a principal component analysis (PCA) aiming to reduce morphological data set into independent components followed by LDA on interpretable PCs. Since the eigenvalues of all principal components were less than one except PC 1 and the broken stick choose only the first principal component (PC1), we choose all the principal components (7 and 5 components for males and females, respectively) that covered about 80% of all the variations following O'Rourke and Hatcher (2013). To remove allometric size-dependent variations, we followed Reist (1986) by log transforming mensural data and regress them against a log-transformed SVL and using resulting residuals in the statistical analyses. Adult male and adult female datasets were analyzed separately.

Geographic distribution data and analysis. Along with molecular delimitation, clear demarcation of the distribution boundaries of a species is a useful tool for the conservation of a species. Primarily, we utilized our GPS records of individual specimens for species distribution analyses and the collection database of *Phrynoidis* specimens at the Field Museum (FMNH). We also leveraged secondary data mining techniques by extracting occurrence data (latitudes and longitudes) from published articles and projected them on Google Earth map to record elevation of the occurrences. We also used published elevation data from articles, if available. Additionally, we used the citizen science data platform iNaturalist.org (https://www.iNaturalist.org) to extract observation data of *Phrynoidis* species

and their elevation. We only used research-grade occurrences with geotagged photographs. However, even in some of the Research-Grade records, we found species were misidentified, based on geotagged photographs uploaded on the site, and we excluded them from our analysis. After identifying useful Research-Grade *Phrynoidis* occurrence, we used GBIF database (https://www.gbif.org/) to download relevant information of those occurrences (Ueda 2020).

RESULTS

Genetic Divergence and Phylogenetic Analyses. Our initial phylogenetic exploration with maximum likelihood analysis on a fragment of 16S gene (493 bp) revealed three completely distinct evolutionary lineages of *Phrynoidis* with considerably high intergroup genetic variations that warrant each of the lineages as a species. In terms of uncorrected p-distance, genetic divergence among these lineages ranged from 4.7% to 6.3% on this small fragment of the 16S gene. One of the lineages is *P. asper* and sequences of this species that ranged from southern Thailand and Myanmar to Java and from Sumatra to Borneo formed a monophyletic group and showed relatively low to moderate intragroup genetic variation (0.0-2.9%). Another lineage—*P. juxtasper*—is endemic to Borneo has a very low intraspecific genetic variation (0.02-1.3%) and occurs sympatrically with Bornean *P. asper* populations. The third lineage occurs in highlands of Sumatra. The distribution of new lineage partially overlaps with the distribution of *P. asper* altitudinally. The new lineage also shows low intragroup genetic variations (0.06-1.9%) and currently recognized as Sumatran populations of *P. juxtasper*. The Sumatran population considered *P. juxtasper* recovered a sister taxon relationship with the endemic Bornean *P. juxtasper* with high intergroup divergence and high nodal support (Fig 1).

After initial exploration of phylogenetic relationships with a fragment of 16S gene, we further investigated and reconstructed phylogenetic trees with following datasets: a) with full 16S rRNA sequence, b) with complete 12S rRNA sequence, c) complete mitochondrial sequences (12S, 16S rRNA genes with flanking $tRNA^{Val}$), d) individual nuclear genes, and e) a concatenated mitochondrial and nuclear dataset.

Independent maximum likelihood and Bayesian analyses of the 12S rRNA (913 bp), 16S rRNA genes (1403 bp), and concatenated mitochondrial sequences (2386 bp) also recovered three evolutionary lineages with similar topologies and relationships like the initial phylogenetic tree with high nodal supports. Uncorrected p-distance in the three major *Phrynoidis* lineages increased slightly when we analyzed the complete 16S rRNA gene. Uncorrected p-distance was 5.8-6.7%, 5.6-7.1%, and 6.7-7.5% between the Sumatran new lineage of P. *juxtasper* and *P. asper*, between the Sumatran new lineage of *P. juxtasper* and *P. juxtasper* of Borneo, and between *P. asper* and *P. juxtasper* of Borneo respectively. The relationships among major lineages derived from individual nuclear genes remained unresolved.

The maximum likelihood analysis of the supermatrix tree of concatenated mitochondrial and nuclear dataset recovered similar relationships like the previous analyses with high nodal supports (Fig. 2A), but with the Bayesian analysis of the concatenated mitochondrial dataset, the relationship of the new taxon remained unresolved with lower nodal supports (Fig. 2B). Regardless of the placement of the new taxon in the phylogenetic tree, nodal support for the monophyly of the genus *Phrynoidis* remained significant.

Morphological analyses. We did not find significant SVL size differences in the *Phrynoidis* species. Table 1 and 2 shows descriptive statistics of males and females of three species. However, the ratio of parotoid gland width to its length is significantly different (in

males: *P. asper* 0.75±0.10; *P. juxtasper* 0.44±0.05; *Phrynoidis* new species 0.56±0.05 and in females: *P. asper* 0.74±0.11; *P. juxtasper* 0.40±0.05; *Phrynoidis* new species 0.56±0.05). Our principal component analyses recovered 7 components significant eigenvalues and for females it was 5. (Table 3). Together these components account for 82.5% of total variations in males and 80.8% of total variations in females. Figure 3 A and B, and Figure 4 A and B display scatterplots of the first three components from the PCA in females and males respectively.

Loading scores provided in table 3 present the interpretable principal components we used in performing LDA. The discriminant analysis correctly classified 76.56% the original cases of males and 96.67% of the original cases and females (Fig 3 C and 4 C). Both males and females of the new *Phrynoidis* species were incorrectly classified as another species. In females, two *P. asper* males were misclassified as the new *Phrynoidis* (sp. nov.) species. Whereas in males, four *P. asper* were misclassified as *P. juxtasper*, two *P. asper* were misclassified as new *Phrynoidis* (sp. nov.) species and four *P. juxtasper* were misclassified as *P. asper*. No new *Phrynoidis* (sp. nov.) has been misclassified as *P. asper* or *P. juxtasper* in males.

Systematics

Phrynoidis sp. nov.

Figs. 5, 6C, 6D

Holotype. Museum Zoologicum Bogoriense of Amphibian Collection, MZB.Amph.23802 (field number ENS 16876), an adult male. Collected from Pangururan located on the west coast of Samosir island of Province of North Sumatra, Indonesia.

2.50195ºN 98, 78845ºE, 918 m a.s.l. Collected by Muhammed Irfan Lubis, Elijah Wostl, and Irvan Sidik on 19 January 2014.

Etymology. The etymology of the specific name will be decided after we decide the specific epithet of the new species.

Suggested Common Name. New Giant River Toad.

Diagnosis. *Phrynoidis* sp. nov. can be diagnosed from its congeners by a unique combination of characters: (1) a giant species of *Phrynoidis* (adult female growing to 142 mm and males growing to 105 mm); (2) tympanum with very thick supratympanic bony crests; (3) parotoid gland length is always less than twice of its width (parotoid gland width/length = 0.56±0.05); (4) at least one phalanx of the fourth toe free of webbing; (5) width parotoid gland is about 40% of the length of its tarsus length (in males = 0.43±0.03; in females = 0.45±0.05).

Description of holotype. Adult male; body robust; head wider than long, HL/HW = 0.90; head length 32.5% of SVL; head width 36% of SVL; snout length 11% of SVL; *canthus rostralis* round; loreal area tuberculate and vertical; eye length 10% of SVL; pupil circular; snout slightly oval in dorsal and protruding (slightly sloping back towards the mouth) in lateral view; tympanum round with distinct annulus; supratympanic crest very thick; parotoid glands very well developed, triangular with tiny, black and sharp-tipped spikes; parotoid gland width is 53% of its length; interorbital space flat; cranial crests absent; no teeth in jaws; tongue tip oval-shaped and longer than wide; skin of dorsal surfaces heavily tuberculate; most tubercles large with keratinization and black sharp tips; no dorsolateral, paravertebral, or occipital folds; skin on venter with relatively small and round tubercles.

Arms robust; forearm length 24 % of SVL; hand length 26% of SVL; fingers long, relative length of fingers: I < II < IV < III; fingertips slightly swollen and black; skin of forearm heavily tuberculate; inner and outer metacarpal tubercles are very well developed.

Thigh length 41% of SVL; tibia length 44% of SVL; tarsal length 23% of SVL; foot length 40% of SVL; relative lengths of toes - I< II< III<V< IV; toes bearing pads but not expanded than the other part of the toes; feet heavily webbed, webbing formula for the feet: I(0) – (1)II(0) – (2)III(0) – (2)IV(2) – (0)V; toes tips black and slightly swollen.

Measurements (in mm) of holotype. SVL 105.12; HL 34.18; HW 37.8; SNL 11.94; ENL 8.37; NSL 4.99; EYL 10.73; TMW 2.9; TED 4.52; PGL 19.84; PGW 10.35; IND 6.65; IOD 9.65; ICD 15.01; FAL 25.22; HNL 27.6; IMTL 4.66; IMTW 4.05; OMTL 6.57; OMTW 6.69; FML 43.06; TBL 45.8; TRL 24.21; FTL 41.79.

Color of holotype in life.—Dorsal surfaces of head and body and limbs dark brown. The surfaces of parotoid glands, limbs with alternate light yellow, and light black stripes. The cloacal region and flanks are predominantly covered with yellow tubercles. Ventral surface of the abdomen covered with yellow non-spiculated and round tubercles. The gular region with reddish maculations and covered with yellow speculated tubercles. Ventral surfaces of thigh covered with non-spiculated and round yellow tubercles. Ventral surfaces of hand and feet black.

Color of holotype in preservative. The general pattern remained the same in alcohol. The yellow and dark colors turned slightly light in contrast.

Paratypes (17).— MZB.Amph.22409, adult female, Provinci Jambi, Kabupaten Kerinci Gunung Tujuh, along trail to Danau Tujuh, 1.71488ºS, 101.3649ºE; MZB.Amph.23805, adult male, Provinci Sumatera Utara, Kabupaten Karo Air Terjur

Sikulipan, 3.24047ºN, 98.53878ºE; MB.Amph.23807, adult female, Provinci Sumatera Utara, Kabupaten Samosir Vicinity of Tele, 2.54691ºN, 98.61414ºE; UTA A 62415, adult male, Provinci Jambi, Kabupaten Kurinci Trail to Danau Tujuh, 1.70868ºS, 101.36981ºE; MB.Amph.23799, adult female, Provinci Sumatera Utara, Kabupaten Karo Sibayak, Foot of mountain, 3.21462ºN, 98.49793ºE; A63431, adult female, Provinci Sumatra Utara, Kabupaten Sumatera Utara Deli Serdang, 3.26898ºN, 98.53992ºE; MZB.Amph.23800, adult male, Provinci Sumatera Utara, Kabupaten Toba Samosir Gunung Pangulubao, 2.60441ºN, 99.04599ºE; MZB.Amph.23801, adult male, Provinci Sumatera Utara, Kabupaten Madina Slope of Dolokk Malea above Kampung Mompang, 0.975ºN, 99.57959ºE; MB.Amph.23904, adult female, Provinci Sumatera Utara, Kabupaten Karo Gunung Sibuatan, Above Kampung Naga Linga, 2.91329ºN, 98.46091ºE; UTAA 63434, adult male, Provinci Sumatera Utara, Kabupaten Samosir W. coast of Island S. of Pangururan, 2.45681ºN, 98.8438ºE; MZB.Amph.23802, adult male, Provinci Sumatera Utara, Kabupaten Samosir W. coast of Island S. of Pangururan, 2.50195ºN, 98.78845ºE; MZB.Amph.23810, adult male, Provinci Sumatera Utara, Kabupaten Mandiling Natal Kota Baringen Julu (Batang Gadis National Park), 0.66636ºN, 99.57191ºE; MZB.Amph.25746, adult male, Provinci Aceh, Kabupaten Bener Merah foot of Berni Terlong, near Desa Rambune, pantan Pediangah, Tiamang Gagah, 4.76449ºN, 96.78224ºE; UTA A-66079, adult female, Provinci Aceh, Kabupaten Gayo Leus Ise-Ise, 4.24819ºN, 97.18185ºE; UTA A-66080, adult female, Provinci Aceh, Kabupaten Gayo Leus Kampung Ise-Ise, 4.25575ºN, 97.18353ºE; UTA A-66076, adult female, Provinci Aceh, Kabupaten Aceh Tengah Kampung Telege Atu, 4.66071ºN, 96.80031ºE; MB.Amph.25744, adult female, Provinci Aceh, Kabupaten Bener Merah , 4.82623ºN, 96.74841ºE; UTA A-66081, adult female, Provinci Aceh, Kabupaten Gayo Leus Stream Along Road S. (up)from Ise-Ise, 4.22357ºN, 97.18655ºE; UTA A-66038, adult female,

Provinci Aceh, Kabupaten Gayo Lues Road from Kedah to Blangkejeren, 4.00477ºN, 97.18412ºE.

Referred Specimens (14). UTA A-66037-38, UTA A-66075-86.

Comparisons. The combination of considerable size, lack of parietal crests, thick supratympanic crests along with and large parotoid glands separates *Phrynoidis* from all other bufonid species of the region (Fig. 6). In *P. asper* parotoid gland shape varies (round, oval or subtriangular) and its width does not exceed one-half of the length (width/length: in females—0.74±0.11, range 0.63-0.96, n=15; in males—n=32) whereas in *P. juxtasper* parotoid gland is always elongated in shape and its length mostly twice or more of its width (width/length, in females—0.40±0.05, range 0.34-0.51, n=17; in males—0.44±0.05, range 0.34-0.56, n=24). On the other hand, in the new *Phrynoidis* species, the parotoid gland is triangular (mostly scalene triangle) and its length is between the one-half and three fourth of its length (width/length in females—0.56±0.05, range 0.47-0.67, n=11; in males—0.56±0.05, range 0.51-0.61, n=8). The ratio of the parotoid gland to the tarsus length is biggest in the new *Phrynoidis* species (in males: 0.43±0.03 vs. 0.27±0.04 in *P. asper* and 0.32±0.03 in *P. juxtasper*; in females: 0.45±0.05 vs. 0.32±0.05 in *P. asper* and 0.34±0.04 in *P. juxtasper*) (Fig. 7).

Distribution and natural history. Our study shows that the new *Phrynoidis* species inhabit mainly in the highlands (mean 1360±337 m a.s.l.), reaching as high as 1827 m elevations in Sumatra. The new species found as low as 617 m a.s.l. The holotype weighed 100.1 g. We found this new species distributed from the southern part of Sumatra—Lampung to the Northern part of Sumatra—Aceh. Except for Bengkulu, Riau, and Sumatera Barat provinces, we collected specimens of the new species from every province of Sumatra. However, Citizen Science data from the GBIF database confirms its presence in the Sumatera

Barat province. The largest individual we collected was a female (SVL 142.03 mm) from 1981 m a.s.l. of Gunung Sibuatan, above Kampung Naga Linga of Sumatera Utara province and weighed 628.7 g.

DISCUSSION

Phylogenetic studies of genera from Southeast Asia with few species have discovered new taxa in recent years (Hamidy *et al.* 2018, Sarker *et al.* 2019). Our study revealed 5.8% to 7.5% interspecific genetic distance in 16S rRNA gene which is comparable to the other contemporary new bufonid taxa discovered from the region (Waser *et al.* 2017, Smart *et al.* 2017, Hamidy *et al.* 2018, Sarker *et al.* 2019, Matsui 2019). Furthermore, this genetic distance exceeds the acceptable observed values for delimiting anuran species (Vences *et al.* 2005, Fouquet *et al.* 2007). This study asserts findings in previous studies that widely distributed species with specific ecological requirements are cryptic species groups instead of single species. Although there is shallow intraspecific genetic divergence with each of the *Phrynoidis* lineages, some *P. asper* shows relatively moderate (up to 2.9%) genetic variations in the 16S gene. Also, we found *P. asper* specimens from East Java are morphologically bigger. These observations underscore the need for extensive geographical, morphological, and molecular studies of *P. asper* to explore species boundaries of this species and if multiple species are masquerading as *P. asper*.

Inger *et al.* (2017) demonstrated *P. juxtasper* is distributed in the forest areas from the sea level to 1600 m a.s.l. in all parts of Borneo, however, it is most abundant in the easternmost Sabah province of Malaysian Borneo. We found similar kind of asymmetry in the distribution of the new *Phrynoidis* species in Sumatra where it is abundant in the northern Aceh and Sumatera Utara provinces, however, it is found in other provinces of Sumatra and

mostly occurs in the mid to highland forest areas (617 m a.s.l. to 1817 m a.s.l.). Konopik *et al.* (2014) show that *P. juxtasper* is an ant-specialist species, however, its abundance is closely tied with the availability of lotic environment and primary forests but not the availability of ant species or the number of ants. Based on our data and observation, we find new *Phrynoidis* species is also a primary forest dweller and lotic habitat specialist. Given that most of the rain forest in Sumatra is under the threat of deforestation, new species of *Phrynoidis* should be considered as a Near Threatened species and necessary conservation measures should be taken for this species.

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Table 1. Descriptive statistics of males of different *Phrynoidis* species

	P. asper	*P. juxtasper*	*Phrynoidis* New Species
SVL	779±9.85 (59.26-104.87)	108.33±7.69 (86.76-122.88)	96.6±8.24 (84.72-105.87)
HL	27.02±3.58 (20.28-35.72)	34.17±2.54 (26.89-38.95)	33.83±2.21 (31.14-36.91)
HW	28.34±3.97 (19.43-38.18)	38.9±3.29 (30.01-45.33)	36.97±2.87 (32.41-41.16)
SNL	10.39±1.65 (7.55-15.11)	13.91±1.3 (11.36-17.37)	12.07±1.07 (10.19-13.53)
ENL	6.96±1.06 (4.89-9.82)	9.21±0.75 (7.9-10.68)	7.64±0.54 (6.94-8.37)
NSL	3.47±0.61 (2.54-4.99)	4.29±0.63 (2.9-6.21)	4.21±0.38 (3.8-4.99)
EYL	8.82±1.65 (6.1-13.19)	11.13±1.36 (8.79-15.02)	10.89±0.86 (9.24-12.41)
TMW	3.09±0.56 (2.01-4.6)	3.19±0.54 (1.95-4.34)	2.91±0.74 (2.06-4.17)
TED	3.54±0.8 (2.03-5.35)	4.84±0.65 (3.38-5.88)	4.64±1.31 (3.06-7.12)
PGL	7.54±2.12 (5.34-15.34)	20.53±2.23 (17.09-25.07)	18.21±2.28 (15.39-21.87)
PGW	5.57±1.47 (3.63-11.41)	8.99±1.06 (6.61-10.78)	10.23±1.22 (9.03-13.02)
IND	5.75±1.15 (3.82-8.68)	7.79±0.7 (6.45-9.68)	6.85±0.99 (5.79-8.63)
IOD	9.88±2.12 (6.8-15.06)	14.32±1.43 (11.06-16.52)	11.16±2.44 (8.81-16.19)
ICD	12.4±2.57 (8.06-19.19)	17.32±1.69 (13.8-21.63)	14.77±2.27 (11.25-18.82)
FAL	19.72±2.99 (15.01-27.49)	28.25±2.3 (23.07-32.86)	24.04±2.3 (20.62-26.68)
HNL	23.06±2.81 (17.86-31.25)	30.39±2.32 (25.35-35.85)	28.68±1.92 (26.95-31.9)
IMTL	3.54±0.72 (2.49-6.1)	5.66±0.68 (4.51-7.14)	4.67±0.64 (3.69-5.55)
IMTW	2.76±0.69 (1.72-4.64)	3.85±0.51 (2.95-4.83)	3.52±0.62 (2.61-4.24)
OMTL	4.64±0.7 (3.68-6.59)	6.77±0.64 (5.44-7.88)	6.25±0.59 (5.27-7.1)

OMTW	3.82±0.7 (2.4-5.88)	6.17±0.63 (4.21-7.17)	5.95±0.6 (4.79-6.69)
FML	38.95±6.15 (29.45-55.21)	50.99±4.03 (45.12-58.2)	44.2±5.09 (37.44-53.02)
TBL	39.08±4.57 (30.67-52.13)	50.8±3.32 (42.92-57.9)	44.65±2.92 (40.93-48.98)
TRL	20.58±2.81 (16.08-29.6)	27.84±2.41 (22.56-32.83)	23.64±1.35 (22.07-26.11)
FTL	34.66±3.81 (24.02-44.09)	45.1±4.1 (36.11-53.77)	41.23±2.77 (36.88-45.57)

Table 2. Descriptive statistics of females of different *Phrynoidis* species

	P. asper	*P. juxtasper*	*Phrynoidis* New Species
SVL	112.08±18.04 (85.89-155.66)	120.43±31.22 (85.47-183.22)	109.4±32.43 (74.46-176)
HL	35.31±5.11 (27.35-43.81)	37.65±8.01 (28.85-55.4)	36.73±9.86 (25.57-57.95)
HW	39.38±6.55 (29.08-51.45)	44.44±10.96 (32.82-69.76)	41.65±11.47 (29.55-64.82)
SNL	14.24±1.95 (11.21-18.57)	14.99±3.21 (10.68-22.02)	13.01±2.9 (10.02-18.91)
ENL	9.11±1.34 (7.25-11.93)	10.2±2.13 (7.73-14.98)	8.93±1.74 (5.86-11.31)
NSL	4.69±0.79 (3.39-6.35)	4.95±1.17 (3.48-7.28)	4.32±1.04 (2.86-6.54)
EYL	11.04±2.13 (7.89-14.99)	11.87±2.44 (8.75-18.34)	11.21±3.18 (7.47-14.78)
TMW	4.11±0.83 (2.47-5.54)	3.48±0.96 (1.91-5.42)	3.63±1.63 (1.71-7.3)
TED	4.9±0.91 (3.41-6.61)	5.8±1.73 (3.92-10.3)	5.81±1.61 (3.89-8.74)
PGL	11.53±3.27 (7.64-20.14)	26.56±8.13 (17.66-43.19)	21.06±7.57 (13.26-37.24)
PGW	8.4±1.99 (5.88-12.1)	10.58±3.18 (6.2-16.57)	11.63±3.6 (7.86-19.73)
IND	7.93±1.15 (5.95-10.04)	8.42±2.19 (5.76-13.74)	7.93±1.58 (5.58-11.04)
IOD	12.53±2.73 (8.51-18.61)	16.58±4.1 (12.56-25.2)	13.8±2.74 (9.99-17.79)
ICD	17.73±2.63 (13.95-23.37)	19.99±4.83 (14.45-29.65)	16.63±3.41 (12.99-23.43)
FAL	27.7±3.38 (23.18-35.17)	31.1±7.43 (22.52-47.04)	26.34±5.71 (20.59-38.99)
HNL	31.47±4.41 (24.49-40.57)	35.03±8.28 (25.61-52.54)	31.73±8.22 (21.38-47.16)
IMTL	5.06±1.01 (3.68-7.38)	6.68±1.69 (4.4-10.13)	5.04±2.16 (2.91-9.27)
IMTW	4.13±0.65 (3.24-5.61)	4.3±1.71 (2.66-7.86)	3.95±1.28 (2.17-6.36)
OMTL	6.81±1.18 (5.12-9.47)	8.08±2.02 (4.98-12.4)	7.62±2.52 (4.96-12.04)

OMTW	5.37±1.01 (4.18-7.92)	7.01±1.97 (4.46-10.31)	6.37±2.59 (3.69-11.25)
FML	51.93±7.43 (41.73-68.18)	55.95±15.08 (35.89-84.51)	49.98±11.09 (38.98-71.85)
TBL	51.81±4.7 (44.95-62.18)	57.78±12.36 (43.72-82.27)	48.89±9.97 (37.34-68.21)
TRL	26.35±3.13 (20.1-34.05)	30.87±6.79 (24.11-43.73)	26.26±7.43 (18.46-41.42)
FTL	47.39±4.99 (40.76-57.17)	51.19±12.21 (38.05-78.42)	47.17±11.62 (33.76-71.19)

Table 3. Loading scores of variables onto the significant principal components used for linear discriminant analysis (LDA)

	Males							Females				
	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7	PC 1	PC 2	PC 3	PC 4	PC 5
HL	0.049	0.036	0.011	0.095	-0.181	-0.034	0.168	0.008	0.094	0.022	-0.115	-0.154
HW	0.045	0.015	-0.014	0.079	-0.126	-0.027	0.108	0.060	0.083	0.014	-0.077	-0.107
SNL	0.037	0.152	-0.055	0.101	0.104	0.151	0.223	-0.017	0.106	-0.050	0.005	0.169
ENL	-0.014	0.066	-0.080	0.064	0.223	0.237	0.189	0.042	0.044	-0.035	-0.039	0.279
NSL	0.057	0.108	0.025	0.422	-0.152	0.626	-0.022	-0.036	-0.044	-0.140	0.247	0.152
EYE	0.043	0.052	0.216	0.182	-0.186	-0.290	0.572	0.001	0.027	-0.018	-0.283	-0.239
TMW	-0.248	0.258	0.883	-0.039	0.032	-0.036	-0.199	-0.207	0.696	0.308	-0.222	0.181
TED	0.110	0.546	-0.168	-0.317	-0.282	-0.097	-0.206	0.170	-0.108	0.338	0.399	-0.055
PGL	0.758	-0.223	0.227	-0.231	0.200	-0.001	-0.130	0.799	-0.043	-0.117	-0.142	-0.089
PGW	0.454	0.150	0.095	0.205	-0.429	0.166	-0.069	0.295	0.032	0.604	-0.005	-0.108
IND	0.019	0.304	0.044	-0.009	-0.097	0.103	0.112	0.009	0.000	0.079	0.074	-0.072
IOD	0.105	0.463	-0.151	-0.281	0.277	-0.039	0.035	0.300	0.080	0.055	0.285	0.486
ICD	-0.008	0.267	-0.016	-0.005	0.040	0.029	0.169	0.048	0.026	-0.087	0.087	0.214
FAL	0.035	0.070	-0.001	-0.038	0.137	0.126	0.022	0.035	0.157	-0.154	0.132	0.128
HNL	0.053	0.006	0.054	0.024	-0.035	-0.082	0.134	0.049	0.172	0.007	-0.048	0.083
IMTL	0.192	0.057	0.136	0.187	0.521	-0.013	0.273	0.160	0.304	-0.567	0.038	-0.205
IMTW	0.076	0.247	-0.146	0.649	0.218	-0.435	-0.384	-0.050	0.354	-0.019	0.624	-0.475
OMTL	0.091	0.034	0.001	0.134	0.037	-0.104	-0.172	0.128	0.222	0.064	-0.119	-0.140
OMTW	0.242	-0.060	0.025	0.012	-0.236	-0.277	0.164	0.213	0.282	0.006	-0.194	-0.081

FML	0.023	0.226	-0.062	-0.037	0.088	-0.020	0.240	0.027	0.137	0.011	0.199	0.127
TBL	0.027	0.094	0.002	-0.033	0.084	0.064	0.116	0.039	0.124	-0.112	0.068	0.202
TRL	0.088	0.076	0.053	-0.033	0.170	0.260	-0.132	0.090	0.117	-0.092	-0.047	0.234
FTL	0.035	0.051	0.009	-0.049	0.005	-0.143	0.124	0.023	0.117	0.023	0.081	0.085

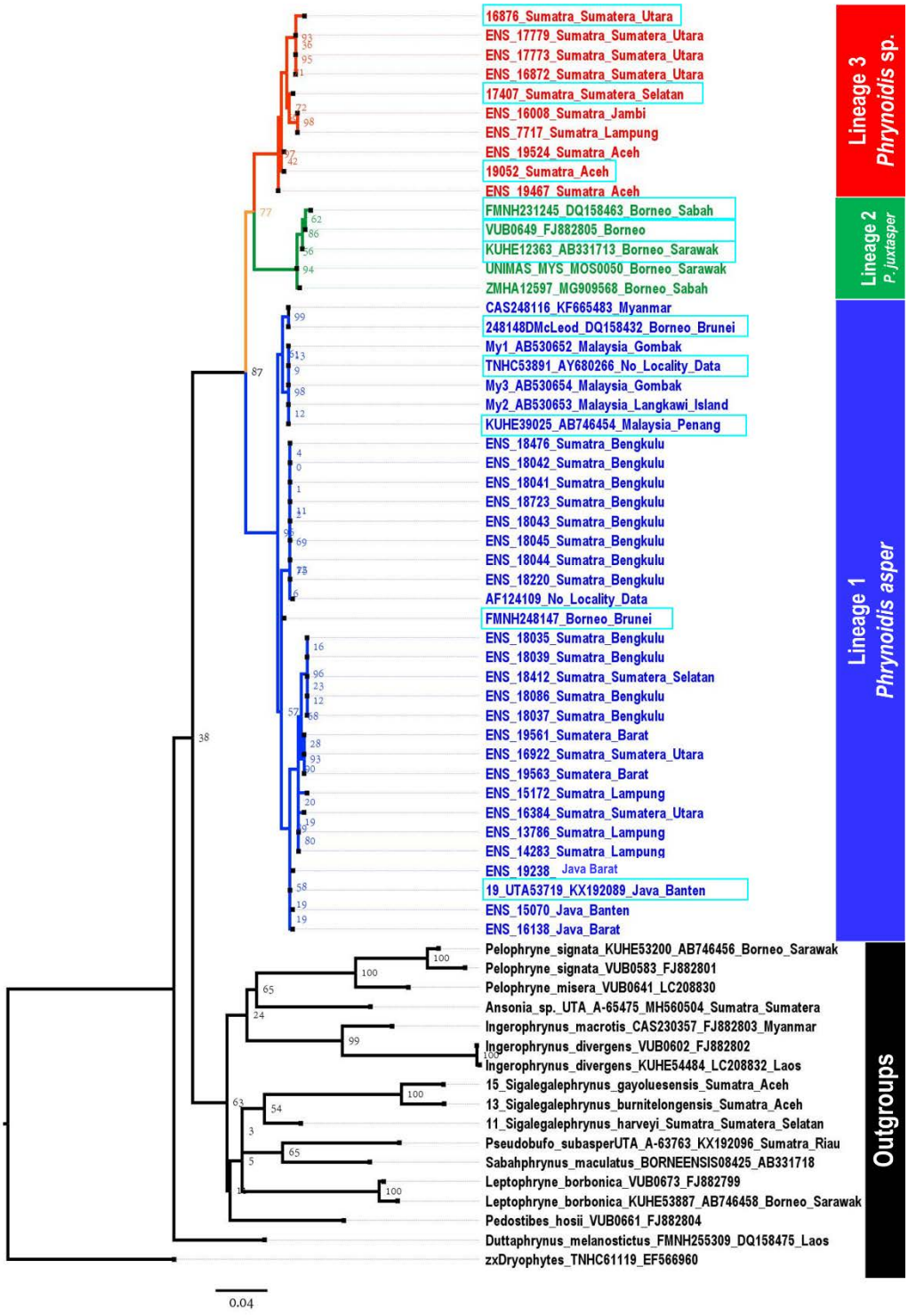

Figure 1. The maximum likelihood tree base on one fragment of the 16S rRNA gene (496 bp) reveals three distinct clades of *Phrynoidis*. Taxa highlighted with cyan box used for further analysis with concatenated and additional mitochondrial and nuclear genes.

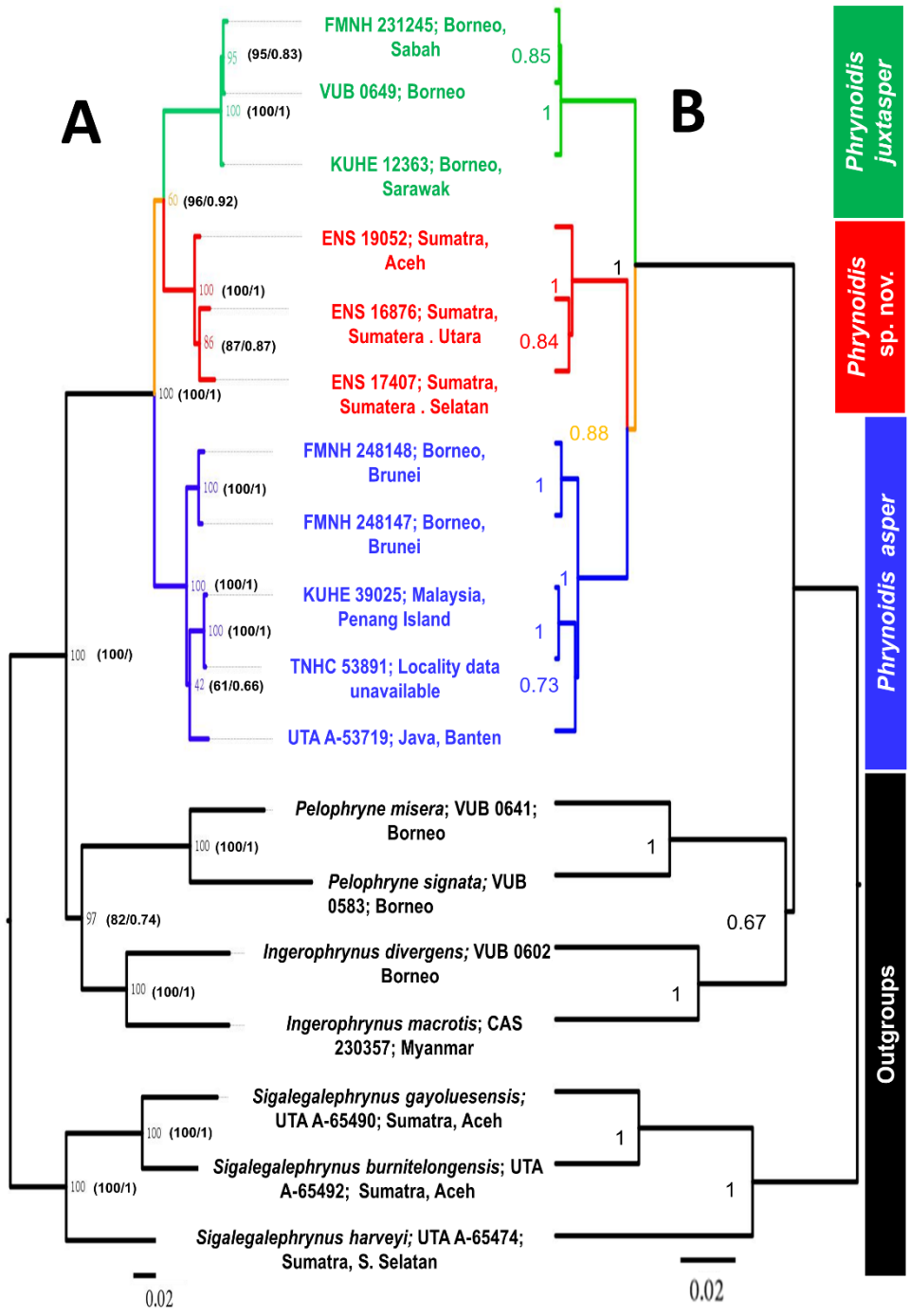

Figure 2. A) Phylogenetic tree derived from maximum likelihood analysis of the full mitochondrial gene (nodal support values outside parenthesis), an identical relationship also derived from the super-matrix concatenated mitochondrial and nuclear genes (ML and BI nodal support values inside parenthesis); B) Bayesian tree of concatenated mitochondrial genes only.

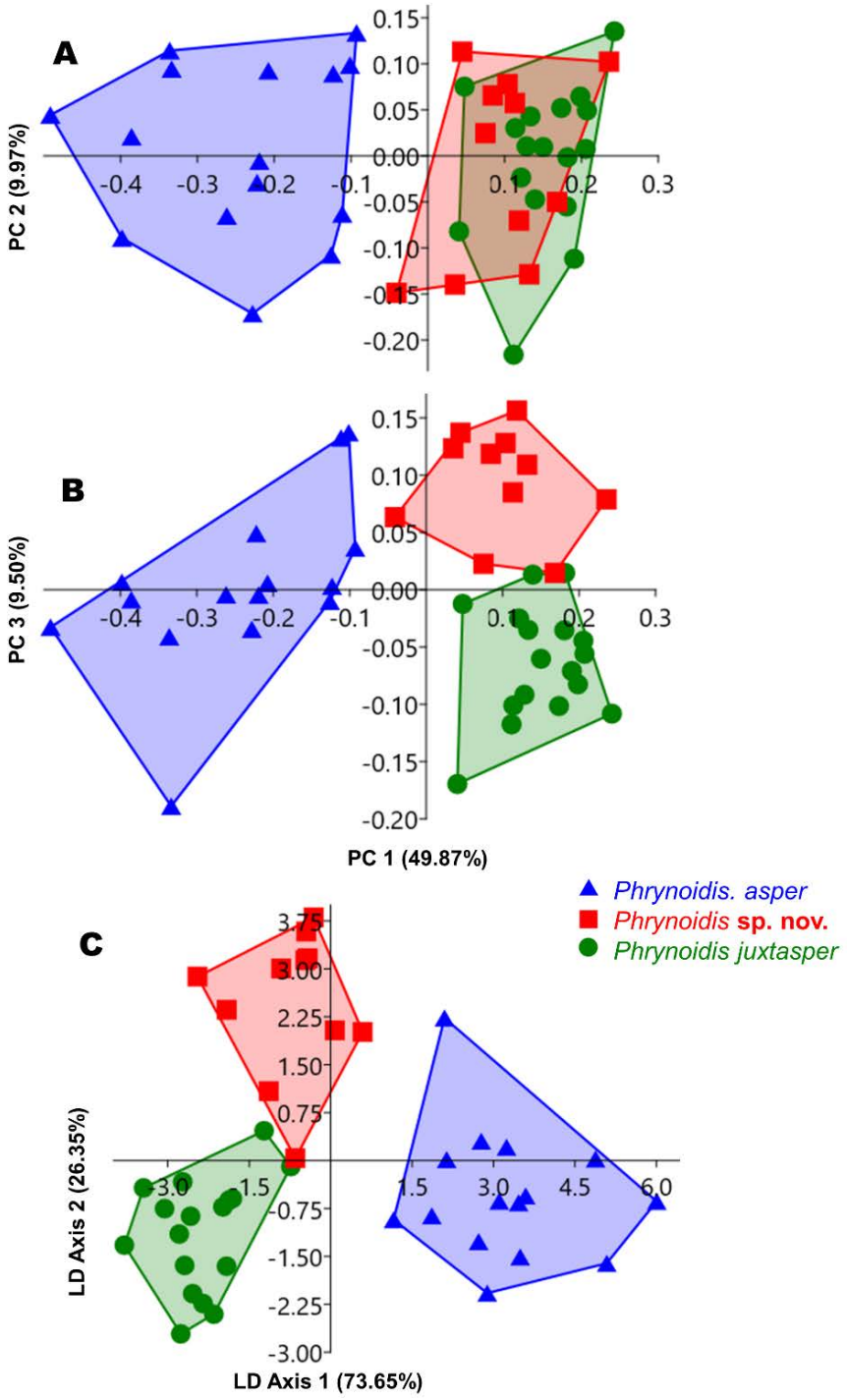

Figure 3. In females: A & B) Scatter plots of the first three principal components showing the distribution of three species in the morphospace, C) plot of discriminant analysis of significant principal components shows complete separation.

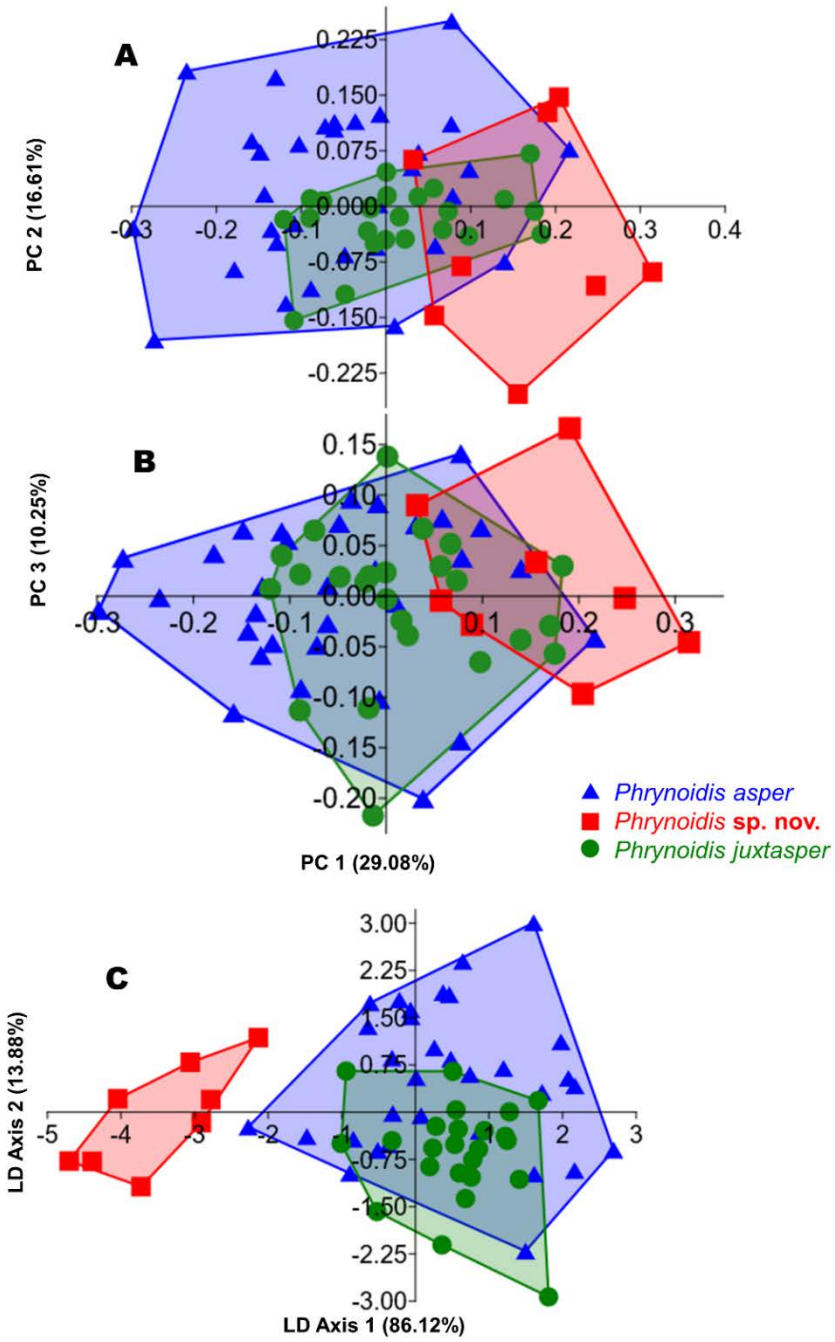

Figure 4. In Males: A & B) Scatter plots of the first three principal components showing the distribution of three species in the morphospace, C) plot of discriminant analysis of significant principal components shows complete separation.

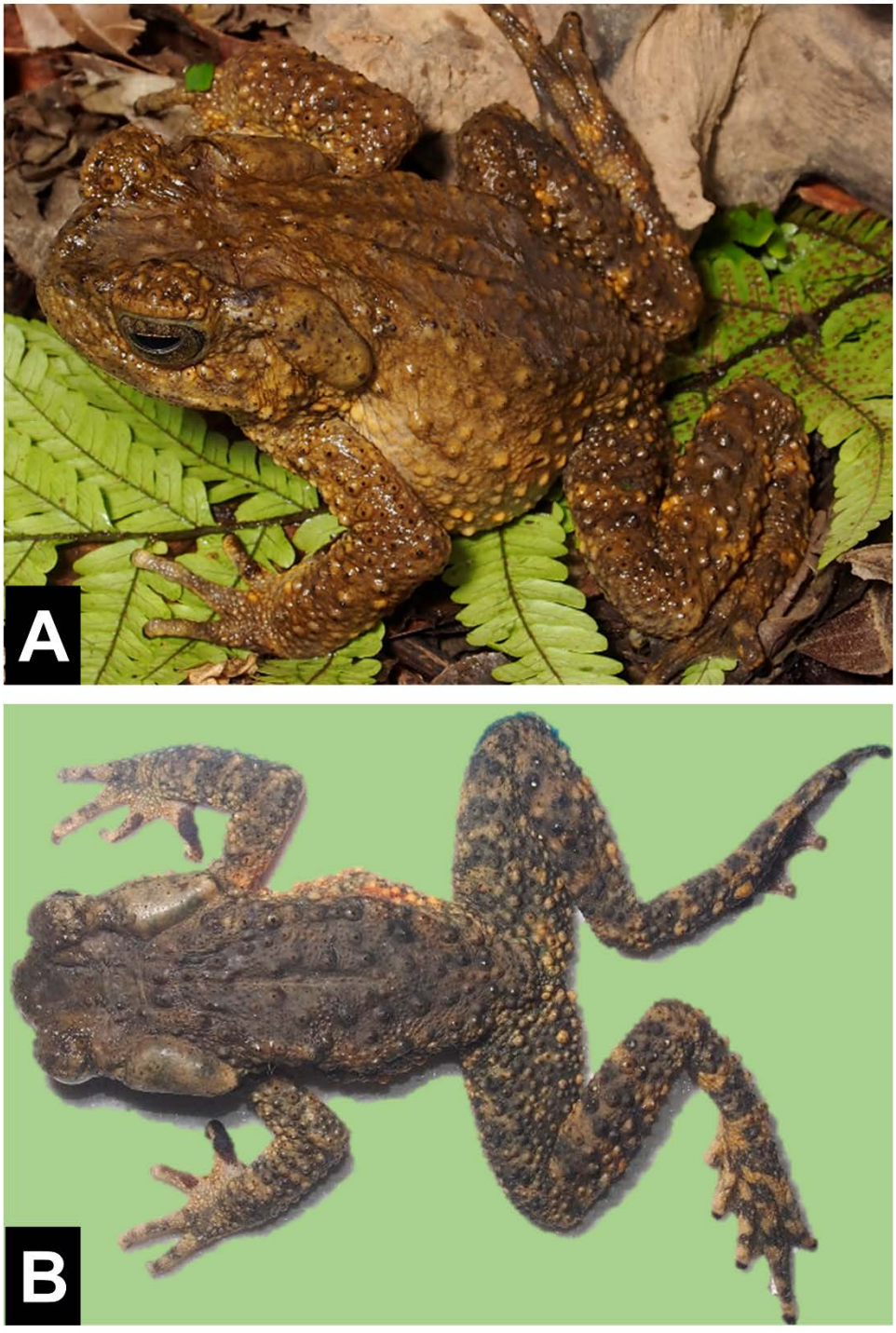

Figure 5. Photograph of new *Phrynoidis* species: A) MZB.Amph.23810—paratype in life, B) MZB.Amph.23802—holotype immediately after euthanization.

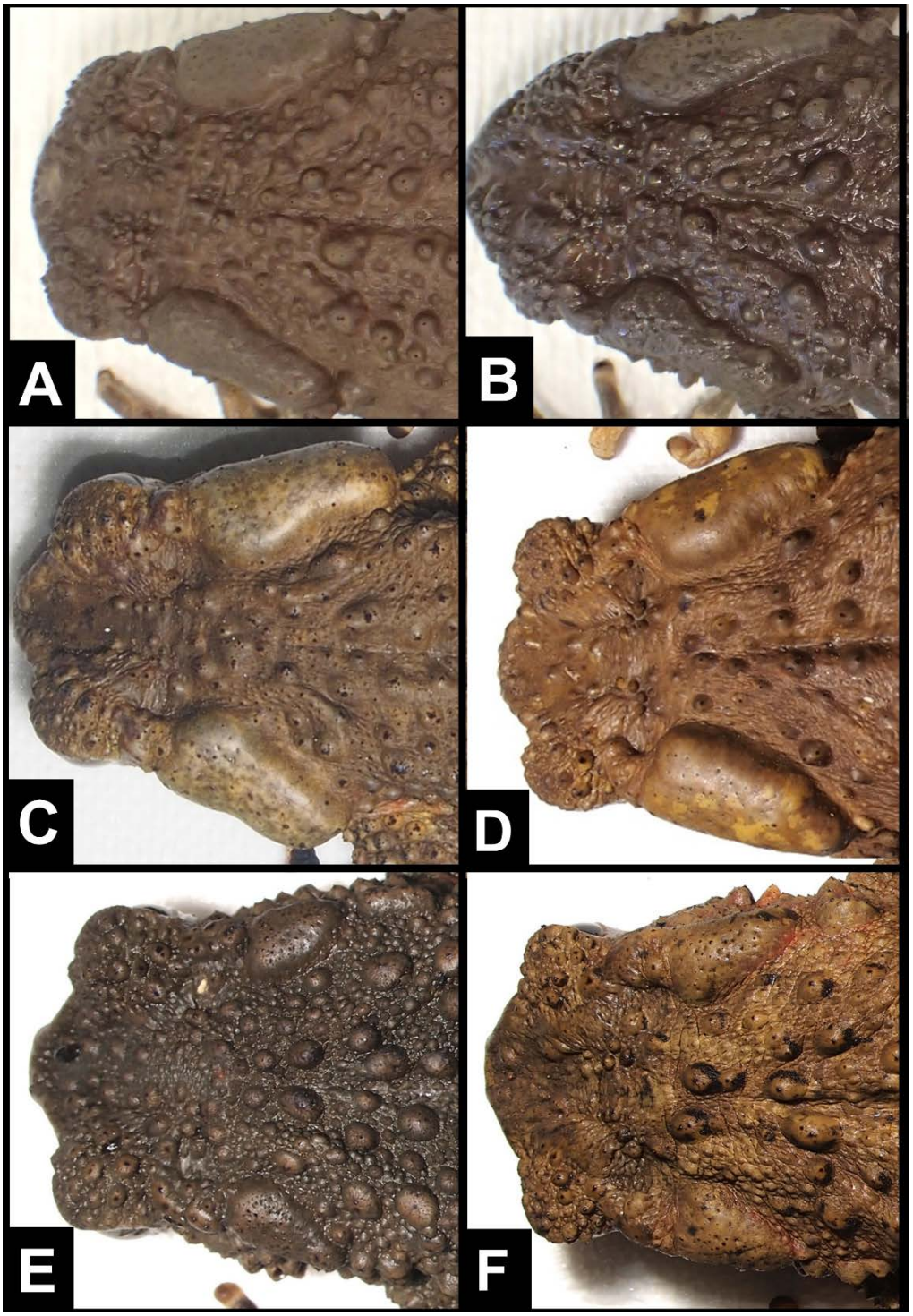

Figure 6. Photographs of *Phrynoidis juxtasper* (A—male, B—female); New *Phrynoidis* species (B—male, C); and *Phrynoidis asper* (E, F) showing distinctive shape and size differences of parotoid glands in different species of *Phrynoidis*.

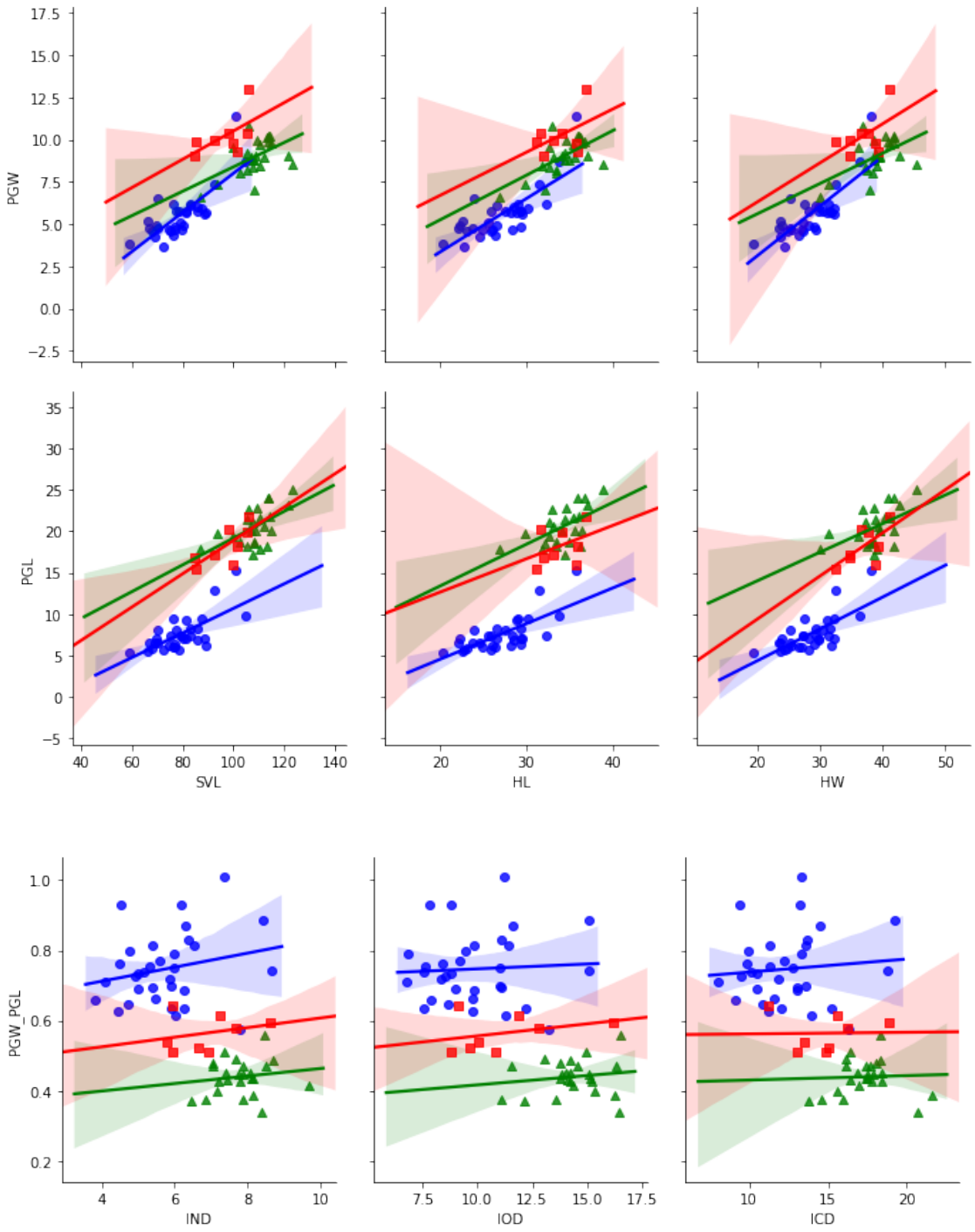

Figure 7. Scatter plots shows relationship of relative length of parotoid gland width (top row), parotoid length (middle row) and ratio of parotoid gland width to its length to other measurements (blue=*P. asper; green = P. juxtasper*; red = *P.* new species).

CHAPTER FIVE

From Siam to Java: Genetic diversity and biogeography of the genus *Leptophryne* (Anura: Bufonidae) emphasizing conservation challenges for a cryptic species complex

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ABSTRACT

Southeast Asian bufonid widely distributed species found across the major Sunda islands and the Malay peninsula have proven to be cryptic species complexes rather than a single species. *Leptophryne* is one of those genera with a widely distributed species, *L. borbonica*. This taxon as currently recognized is found from southern Thailand to Java and from the islands of Siberut and Nias to Borneo. We investigated the molecular diversity and phylogenetic relationships of populations of this widespread species using mitochondrial DNA. We identified five distinct and independently evolving lineages within *L. borbonica*, that contained multiple sub-lineages. We find *L. borbonica* to be a species complex of at least five species maskarading within a single taxon, and point to the need of further DNA and morphological investigation. These findings also underscore the need for reevaluation of two species junior synonyms—*Nectophryne sumatrana (=Leptophryne sumatrana)* and *Bufo jerboa (=Leptophryne jerboa)*—and the possibility of their recognition.

INTRODUCTION

The genus *Leptophryne* Fitzinger, 1843, consists of three species, *Leptophryne borbonica* (Tschudi 1838), *L. cruentata* (Tschudi 1838), and *L. javanica* Hamidy, Munir, Mumpuni, Rahmania, and Kholik, 2018. The latter two are sister species and point endemics to Java. *Leptophryne cruentata* is restricted to the northern slopes of mountaintops between 1000 m and 2000 m a.s.l of Gunung Gede and Gunung Halimun in West Java (Liem 1971, Iskandar 1998, Kurniati 2003, Kurniati *et al.* 2006, Kusrini *et al*. 2016, Frost 2020). *Leptophryne javanica* is restricted to Gunung Ciremai and Gunung Slamet between 1200 m and 1500 m a.s.l. in central Java (Hamidy *et al.* 2018). In contrast to *L. cruentata* and *L. javanica*, *L. borbonica* is a widely distributed species that ranges from the Thai Isthmus of Kra and the

Malay Peninsula to the greater Sundas, Borneo, Sumatra, and Java, its type locality (West Java; Inger 1966, Frost 2020). According to the IUCN Red List, *Leptophryne cruentata* is Critically Endangered and the recently described *L. javanica* is Data Deficient. On the other hand, the widely distributed species—*L. borbonica* is placed under the "Least Concern" category due to its "vast" range, despite populations declining and facing various threats of extinction, e. g., deforestation, habitat alteration, and urbanization (AmphibiaWeb 2020).

The taxonomy of the genus *Leptophryne* and its species has remained controversial for more than a century. Schlegel (1826) first described *Leptophryne borbonica* as *Hylaplesia borbonica* without any description of the species, resulting in a *nomen nudum*. However, Tschudi (1838) gave a formal description of the species and described a second species—*H. cruentata*. Cope (1867) transferred *Hylaplesia borbonica* to the genus *Bufo* and synonymized both *H. borbonica* and *H. cruentata* as *B. borbonica*. Boulenger (1890) accepted the synonymy and identified one population of *Leptophryne* from Southeast Borneo and described this as *B. jerboa.* In 1910, van Kampen discovered one population of *Leptophryne* from Bandar Baru, about 1000 m in levation near the city of Medan, Sumatera Utara, and described it as *Nectophryne sumatrana.* In 1912 he transferred *L. borbonica* to the genus *Nectophryne.* Smith (1930) examined a large series of *L. borbonica* from Southwest Phatthalung (= Patelung), Thailand, and synonymized *Bufo jerboa* (= *L. jerboa*) with *B. borbonica.* Davis (1935) transferred all the species to the genus *Cacophryne*. Inger (1966) mentioned significant size variation of different populations of *L. borbonica* and examined the type specimen of *Bufo jerboa*, from Borneo, and synonymized this as *Cacophryne borbonica*. Coincidentally, both Davis (1935) and Inger (1966) themselves did not examine the type specimen of *L. sumatrana* (= *Nectophryne sumatrana*). In our study, we took the opportunity to examine all the type specimens of *L. borbonica* and its junior synonyms (*B.*

jerboa and *N. sumatrana*) to assess the taxonomic position of the populations of *L. borbonica*.

In recent years, studies leveraged molecular data to study and delimit the genus *Leptophryne* along with all other bufonid genera. Previous molecular studies on Southeast Asian bufonids including the genus *Leptophryne* used genetic sequences from no more than seven *L. borbonica* specimens (Hamidy *et al.* 2018, Chan and Grismer 2019). However, these studies discovered moderate to relatively high molecular diversity among different populations of this species and noted the presence of cryptic species diversity. In this study, we address these sampling shortcomings and expanded our molecular sampling to 81 specimens from localities ranging from peninsular Malaysia, Borneo, Sumatra and Java (Fig. 1). With more molecular geographic representation, from dense population sampling across Sumatra and Java, we take the opportunity to do a comprehensive evolutionary assessment of the *L. borbonica* species complex.

MATERIALS AND METHODS

Taxon sampling and DNA sequencing. Specimens were collected in Java and Sumatra from January 2013 to November 2016. Collecting, handling, and euthanizing of specimens strictly followed protocols approved by the UTA Institutional Animal Care and Use Committee (IACUC; number UTA IACUC A12.004). Overall, we collected 114 *Leptophryne* specimens. We took photographs of some specimens in life and of all specimens immediately after euthanasia, recording dorsal, ventral, and lateral aspects. Specimens were fixed in 10% formalin and then transferred to 70% alcohol for permanent storage. Before fixation, we took liver or muscle tissue samples and preserved them in 1.5 mL of cell lysis buffer solution (0.5 M Tris/0.25% EDTA/2.5% SDS, pH = 8.2). Finally, we deposited all specimens at the

Laboratory of Herpetology in the Museum Zoologicum Bogoriense (MZB), and the Amphibian and Reptile Diversity Research Center (ARDRC) of the University of Texas, Arlington (UTA). Museum acronyms used in this manuscript follow Sabaj Perez (2016).

We sequenced one fragment of the 16S mitochondrial gene from 70 *Leptophryne* specimens. Primers and PCR protocol, we followed for this research project can be found in Sarker *et al.* (2019). PCR success was visually assessed on a 1% agarose gel. DNA extraction and PCR products were purified with Serapure beads (following the Agencourt protocol, Beckman Coulter Co., Fort Collins, CO, USA) after Rohland & Reich (2012). The Genomic Core Facility at the University of Texas at Arlington completed the sequencing reactions with an ABI PRISM 3100xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Our molecular sampling includes a fragment of the 16S gene from 81 *Leptophryne* specimens, including 11 sequences from the GenBank. For initial phylogeny exploration and test of monophyly we used at least one representative species from each extant Southeast Asian bufonid genus as ingroup and one New World bufonid—Cayenne stub foot toad (*Atelopus flavescens*), and one hylid frog—Canyon Tree frog (*Dryophytes arenicolor)*. All sequences generated for this project will be submitted to GenBank upon publication.

Sequence alignment, phylogeny inference, and haplotype networks. Assembling and editing of raw sequences were done in Sequencher v 5.3 (Gene Codes, Ann Arbor, MI, USA). Alignment of sequences was performed in MEGA (v6.0; Tamura *et al*. 2013) using the ClustalW algorithm (Larkins et al. 2007) with default parameters. We used both maximum likelihood (ML) and Bayesian inference (BI) methods for phylogeny inferences by using RAxML v8.2.12 (Drummond and Rambaut, 2007) and BEAST v2.6.1 (Suchard and Rambaut 2009) software respectively implemented on the CIPRES gateway server (Miller *et al.* 2010).

We performed ML phylogenetic analysis employing a rapid bootstrapping algorithm using the program RAxML-HPC BlackBox v8.2.10 (Stamatakis 2014) and considered nodes to be strongly supported when having bootstrap values above 70% (Hillis & Bull 1993). For Bayesian phylogeny inference, five independent runs with a chain length of 100 million generations and a log and tree frequency of 10000 generations were performed. We used a relaxed lognormal clock with constant size growth coalescent tree prior, and site models selected using bModelTest plugin (Bouckaert and Drummond 2017). Tracer v1.7.1 (Rambaut *et al.* 2018) was used to visualize and compare convergence and stationarity of each of these individual runs. We then combined them using LogCombiner v.10.4 and annotated our trees using TreeAnnotator v1.10.4 (Drummond and Rambaut 2007) by removing 25% of initial samples as burnin. We considered nodal support with PP values ≥ 0.95 as significant (Huelsenbeck & Rannala 2004; Mulcahy *et al.* 2011). Finally, we used FigTree v1.4.3 (Rambaut, 2012) for graphical visualization of the resulting ML and Bayesian trees.

We used the ML tree generated from the RAxML analysis and trimmed alignment from MEGA X (Tamura *et al.* 2018) to visualize evolutionary relationships among haplotypes by making networks in Haploviewer (Ewing 2011; available from http://www.cibiv.at/~greg/haploviewer). GenGIS software was used for graphical representation of the geographical sampling distribution of *Leptophryne* species (Parks *et al.* 2009).

Divergence time estimation. Using 16S mitochondrial DNA we estimated divergence times of populations, using BEAST 2.6.1 implemented in the CIPRES scientific gateway. We used a secondary calibration following Grismer *et al.* (2017), and to be more conservative we used a 2% divergence per million years to calibrate our molecular clock. TRACER 1.7.1 (Rambaut *et al.* 2018) was used to assess runs and examine convergence. We used a 10%

burnin, discarding 1000 trees, and the remaining trees were used to create a final maximum clade credibility (MCC) tree with median heights.

Species delimitation. We used Poisson tree processes (PTP)—a coalescent-based species delimitation technique to infer the most likely number of species in our dataset, implemented in the Bayesian Poisson tree processes (bPTP) server using both single rate and multi-rate processes (Zhang *et al.* 2013; implemented in https://species.h-its.org/ptp/ and mPTP https://mptp.h-its.org/#/tree respectively). The PTP model is a fast method that uses a robust algorithm of two independent Poisson process categories (within- and among-species substitution numbers) without the requirement of an ultrametric tree.

As for the input file, we used the Bayesian tree obtained from the BEAST analysis. We ran the PTP analysis for 500000 MCMC generations with a thinning value of 100 and a burn-in value of 20%. We removed the most distant outgroup taxa to improve the species delimitation process. We visually checked the likelihood plot of the delimitation to confirm the convergence of MCMC chains. For the ABGD species delimitation method, we used the web-based platform—https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html with default settings (Puillandre *et al.* 2012). Unlike PTP model, ABGD depends on distribution of calculated *p*-distance (between and among groups) and does not require a phylogenetic tree to delimit groups or species.

RESULTS

Our ML and Bayesian analyses recovered almost identical topologies with high nodal supports where *Leptophryne cruentata* and *L. javanica* are siter taxa showing reciprocal monophyly and being a sister group to a clade of all *L. borbonica* populations (Fig. 2). Within *L. borbonica* five major clades were recovered (identified as A–E). These clades

possees moderate divergence, comparable to the amount of divergence between *L. cruentata* and *L. javanica*. Among major clades uncorrected p-distances of 493 bp of *16S* are presented in table 1, supporting clade differentiation as of species level (Fig. 2). Moreover, each major clade also further subdivided into multiple subgroups with high nodal support and considerable inter-subclade divergence (Fig 3).

Leptophryne borbonica populations from Borneo and lower elevation of Sumatra (mainly from the north of Sumatra—Aceh, Sumatera Utara, and Sumatera Barat) belong to Clade A, where Bornean and Sumatran populations formed independent subclades. Clade B and clade C members came exclusively from higher elevations in Sumatra (>1200 m a.s.l). Interestingly, clade D contains populations from lower elevations (<1200 m a.s.l) in all of Sumatra (from north to south) and Peninsular Malaysia. *Leptophryne borbonica* populations from java formed a monophyletic group, clade E, however, ML and Bayesian analyses showed discrepancies in their relationships. Maximum likelihood analysis puts populations from western and eastern Java together, placing the central Javan population basal to them. However, Bayesian analysis show West Java populations as basal (Fig. 3).

Divergence time analysis reveals the oldest split between the *L. borbonica* complex and the ancestor of *L. cruentata* and *L. javanica* to date back to the Miocene, about 10 mya (95% HPD interval 0.78–25.76). The divergence between *L. cruentata* and *L. javanica* is more recent and dates back to 3 mya (95% HPD interval 0.16–7.4). Divergence analyses reveal that splits of major clades of *L. borbonica* happened in the Pliocene or close to it (Fig. 3).

In species delimitation, PTP models with a single rate and multi-rate Poisson processes gave very different results; mPTP failed to recognize some outgroup species and identified multiple outgroup species as one. The PTP model was more accurate in identifying

all outgroup species. It delimited four species in the *L. borbonica* complex by putting clade A and clade B together as a single species. On the other hand, ABGD recovered 18 partitions for *Leptophryne* with a prior intraspecific divergence (P) ranging from 0.0010 to 0.0028. However, the recursive partition was stable by supporting seven partitions (five within the *L. borbonica* complex) and identifying each of the *L. borbonica* clades as single species with a prior intraspecific divergence (P) of 0.21544.

Haplotype networks of *16S* show that *L. cruentata* and *L. javanica,* as well as all the major clades of *L. borbonica* share no inter-species or inter-clade allelic haplotypes. However, if we classify all the sequences into two elevational categories, namely high (>1200 m a.s.l.) and low (>1300 m a.s.l.), we find that allelic haplotype sharing of high elevation clades with others occurs less frequently. On the other hand, haplotype sharing among the lower elevation clades happened more frequently (Fig. 5).

DISCUSSION

Leptophryne borbonica is generally considered a low to mid-elevation species distributed below 1400 m a.s.l. (Inger *et al.* 2017; Hamidy *et al.* 2018), however, we discovered several populations in Sumatra which inhabit high elevation forests. About 30% of our *Leptophryne* collections came from forests between 1400 and 1878 m a.s.l.

As previous studies suggest, there are several candidate species in the *Leptophryne borbonica* complex (Hamidy *et al*. 2018, Chan and Grismer 2019). Our study revealed at least five major independently evolving *Leptophryne borbonica* clades. Two of these clades include specimens from near localities where two species were described and later synonymized with *L. borbonica—Bufo jerboa* and *Nectophryne sumatrana*. Our study underscores the necessity of reevaluation of those two synonyms and their possible

resurrection. Despite our best effort, we could not survey and collect specimens from every mountain of Sumatra and Java, so it is highly likely that many more undiscovered populations and probable new species are awaiting discovery. Our work also underscores the challenge of conservation when cryptic species are not recognized. Currently, as a single species, *Leptophryne borbonica* is considered as "Least Concern" under the IUCN Redlist Status owing to its wide geographical distribution (AmphibiaWeb, 2020). Our data clearly show that the current conservation status of this species is inappropriate and needs immediate reevaluation, especially when numbers of threats, including habitat alteration and urbanization, deforestation, chytrid fungus infection, monoculture and mining activities are rampant in the forests where *L. borbonica sensu lato* inhabits (Hamidy *et al.* 2018, Sarker *et al.* 2019) and threats will be different when all cryptic within the taxon get recognized.

Maximum likelihood analysis recovered a sister taxon relationship between West and East Java and placed *L. borbonica* populations of Central java as a basal taxon. However, Bayesian analysis placed populations from West Java as basal to the sister populations of Central and East Java. Haplotype network analysis showed *Leptophryne* populations from Java formed a distinct branch where East Java has two different lowland haplotype and central Java has one haplotype. On the other hand, West Java has distinct highland and lowland haplotypes. Haplotype sharing among the Bornean and northern Sumatran populations in clade-A, and among the South and central Sumatran, peninsular Malaysia, and Javan populations of *L. borbonica* signify dispersal events. On the other hand, almost no haplotype sharing of the higher elevation populations among the *L. borbonica* populations in Sumatra signify the influence of vicariance events and in-situ diversification within the island. Unlike findings of previous studies of Sarker *et al.* (2019) and Shaney *et al.* (2020), where in-situ factors mainly drove diversification, our study demonstrates that in the genus

Leptophryne, and particularly in *L. borbonica*, most probably diversification is both in-situ and ex-situ methods (Fig. 6).

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Table 1. Uncorrected p-distance between major lineages of *L. borbonica*, *L. javanica* and *L. cruentata.*

Clades/Species	Uncorrected p-distance
Clade A - Clade B	3.5-5%
Clade A - Clade C	5.7-7.3%
Clade A - Clade D	6.4-9%
Clade A - Clade E	6.4-10.7%
Clade A - *L. cruentata*	13.7-14.5%
Clade A - *L. javanica*	14.5-15.8%
Clade B - Clade C	6.4-7.1%
Clade B - Clade D	6.4-7.4%
Clade B - Clade E	7.2-10%
Clade B - *L. cruentata*	14.2-16.1%
Clade B - *L. javanica*	14.7-16.7%
Clade C - Clade D	7.1-7.6%
Clade C - Clade E	7.1-8.6%
Clade C - *L. cruentata*	13.4-14.1%
Clade C - *L. javanica*	13.8-14.7%
Clade D - Clade E	3.2-5.3%
Clade D - *L. cruentata*	14.1-15.3%
Clade D - *L. javanica*	14.9-15.8%
Clade E - *L. cruentata*	14.3-16.6%
Clade E - *L. javanica*	14-15.9%
L. cruentata - *L. javanica*	5.3-5.5%

Figure 1. Sampling locations for present molecular studies (white circles). Small black circles represent sampling localities that previous studies used in their molecular studies.

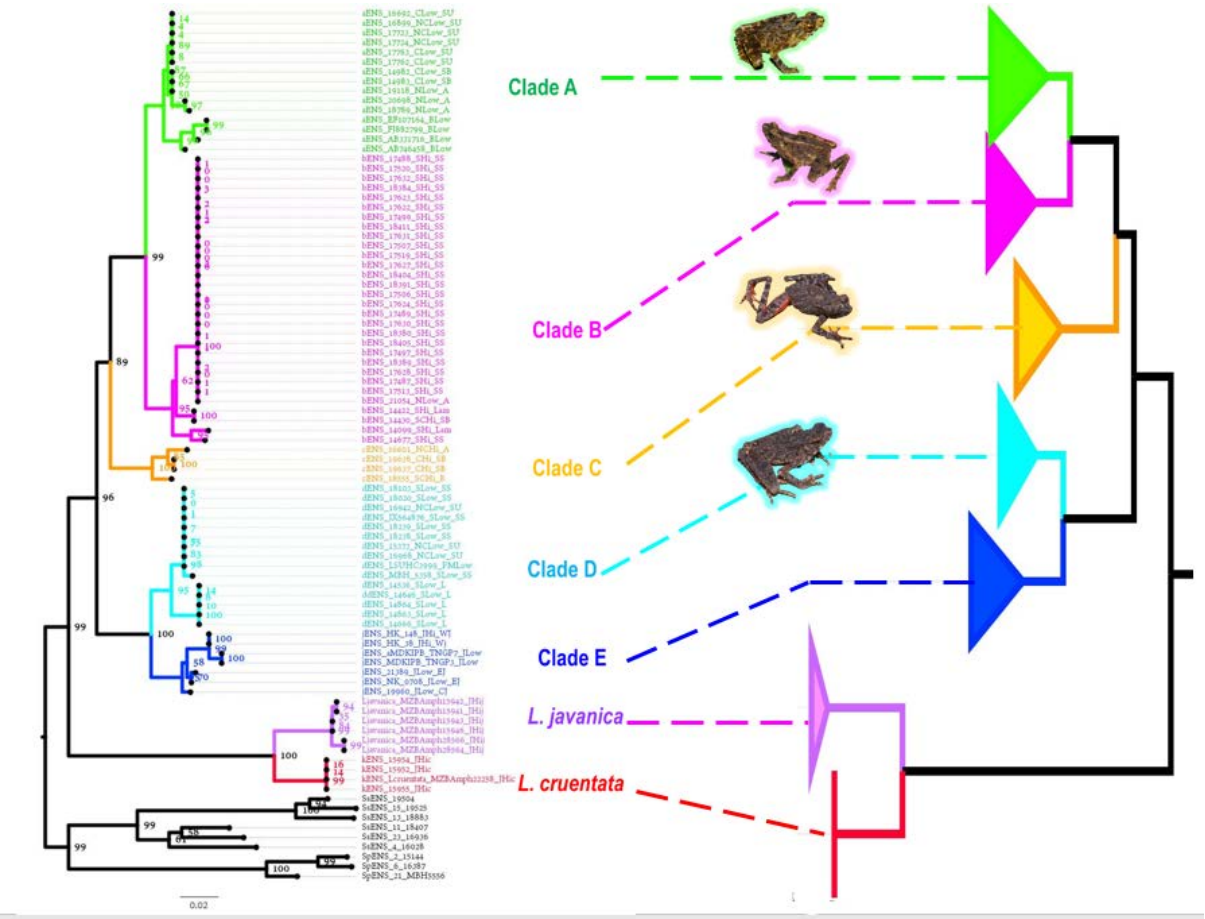

Figure 2. Phylogenetic tree derived from maximum likelihood analysis of one fragment of 16S rRNA gene showing five major clades in the *Leptophryne borbonica* complex.

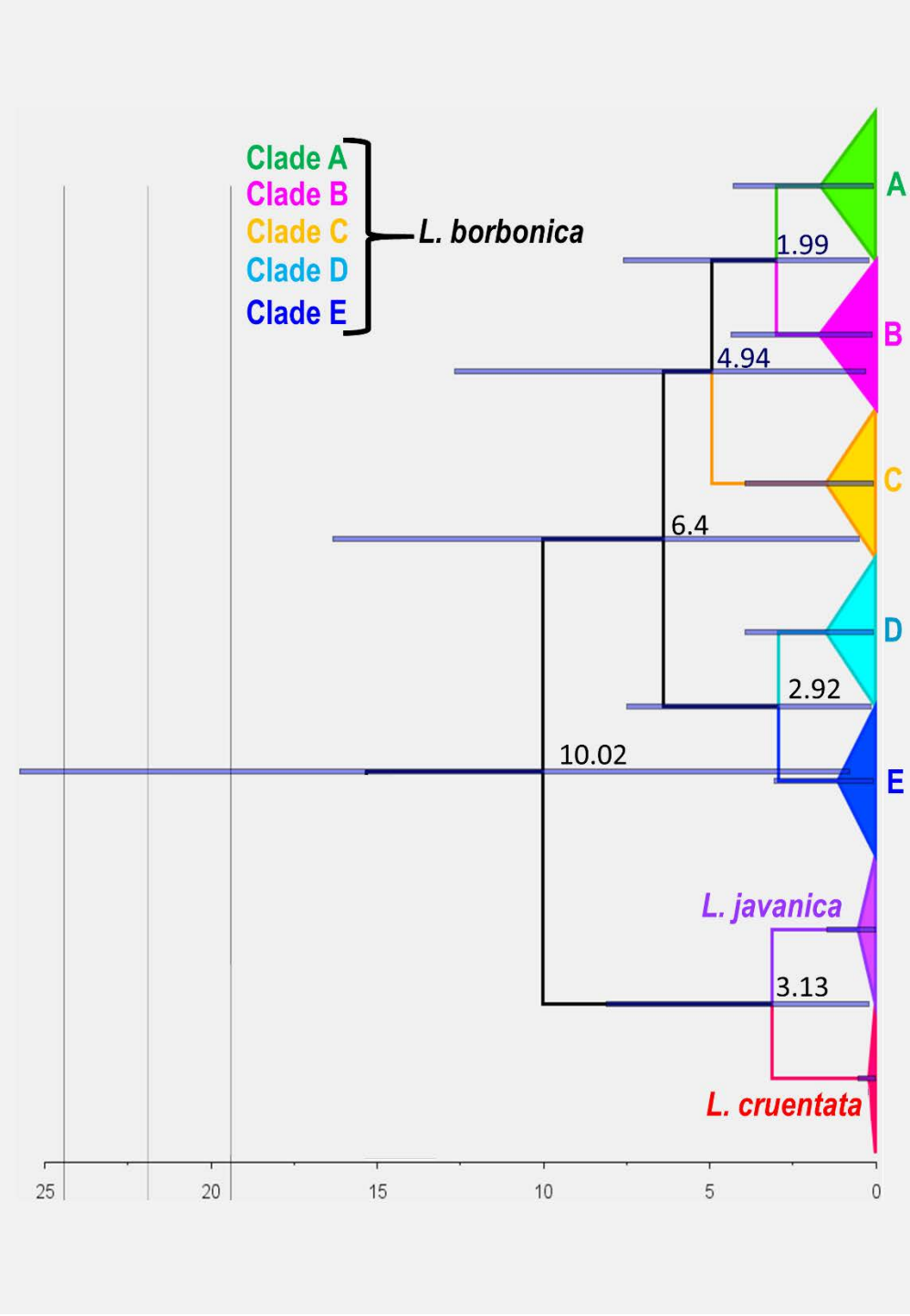

Figure 3. Chronogram of *Leptophryne* species showing the nodal date estimates for the major clades of the *L. borbonica* species complex as well as *L. cruentata* and *L. javanica.*

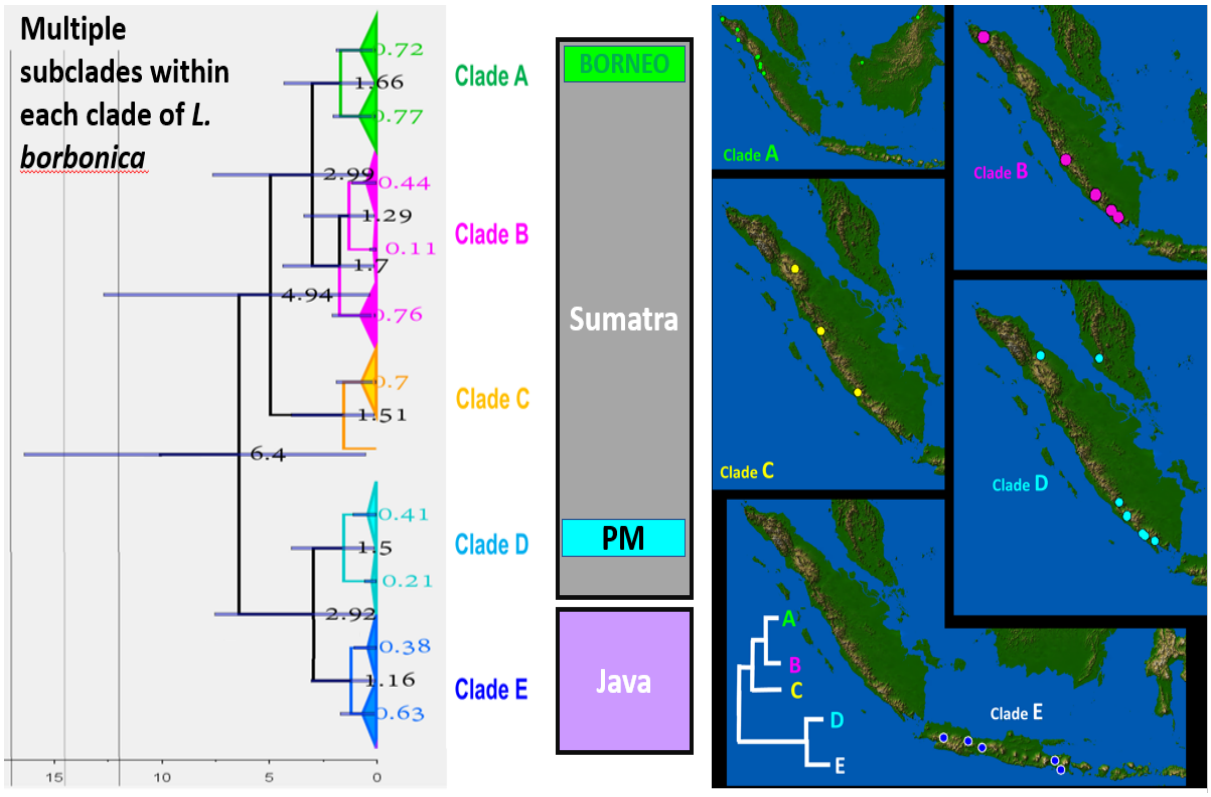

Figure 4. Chronogram of the *Leptophryne borbonica* complex from BEAST analysis on one small fragment of 16S rRNA gene showing subclades within each major clade.

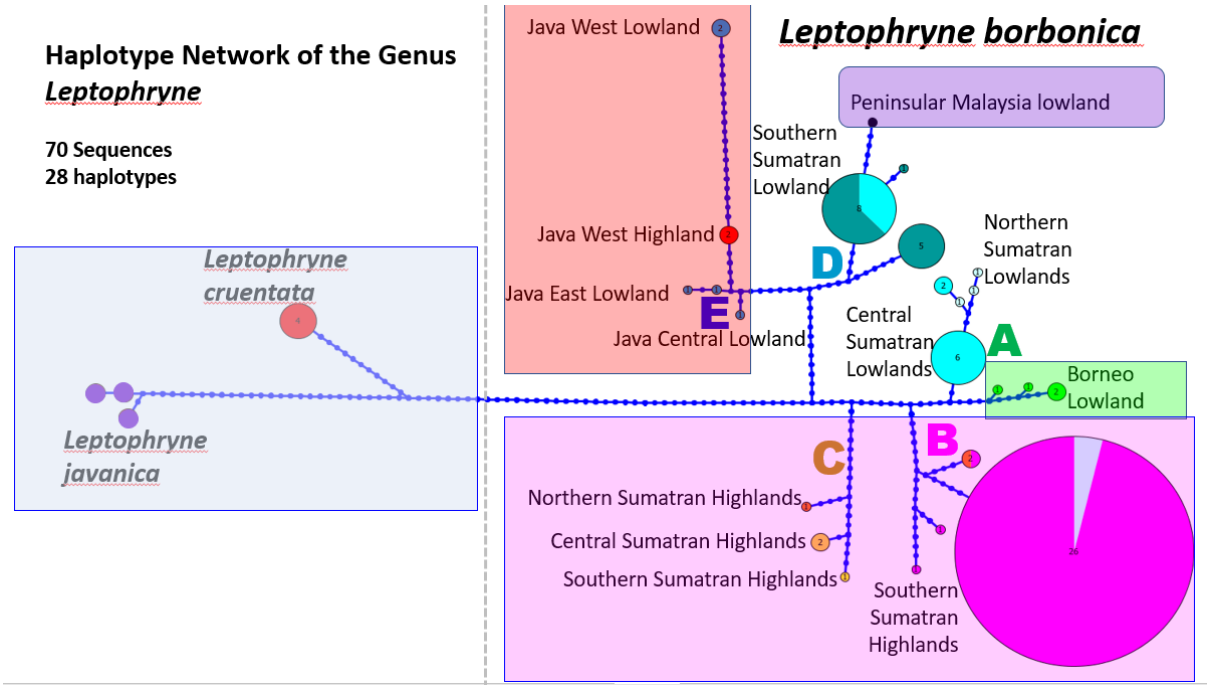

Figure 5. Haplotype (allele) network of a small fragment of *16S* rRNA.

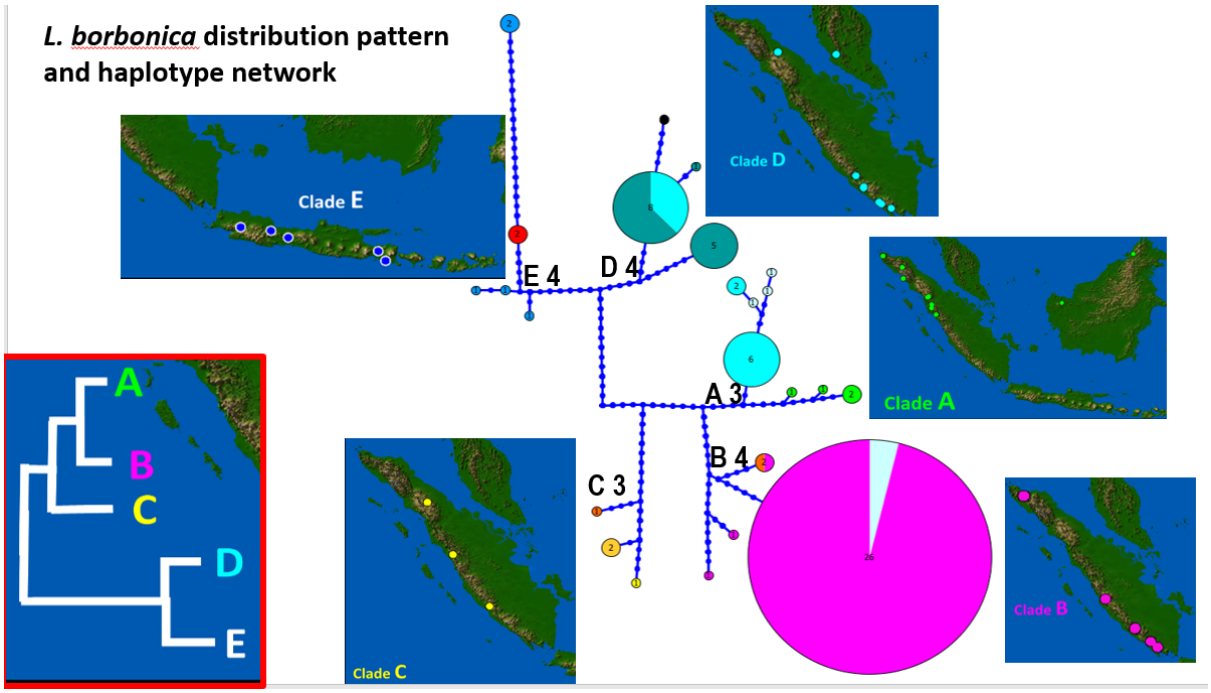

Figure 6. Distribution of *Leptophryne borbonica* and major clades of *L. cf. borbonica* in the Sundaland.

CHAPTER SIX

General Conclusion

The Sundaland is one of the most biodiverse places on earth and considered as a natural laboratory for tropical evolutionary biology studies (Cannon *et al.* 2009, Gower *et al.* 2012). However, like other biological hotspots, the biodiversity of the Sundaland is at grave risk of extinction from a wide range of anthropogenic threats, hence relevant conservation initiatives are in urgent need in the region (Cannon *et al.* 2009, Harvey *et al*. 2017, Hamidy *et al.* 2018, Sarker *et al.* 2019). However, successful conservation and management of wildlife species depend on the proper understanding of the distribution, genetic diversity patterns, correct taxonomic identifications and the assessment of threats of extinction. Unfortunately, much of the Sundaland, particularly Indonesia's major islands—Sumatra, Borneo (Kalimantan), and Java—are the least explored (Brown and Stuart 2002, Harvey *et al*. 2017, Hamidy *et al.* 2018, Sarker *et al.* 2019) and facing the highest rate of deforestation (Margono *et al.* 2014). Thus more studies to uncover Sundaland's biotic diversity, particularly that of the large Indonesian islands, are needed. Although there are many still unsampled mountains and forests in Sumatra, Java, and Kalimantan, this study incorporated the most comprehensive sampling of bufonid frogs after van Kampen's 1923 work on the amphibians of Indonesia. I believe, my study on the bufonid diversity will add useful information to the body of knowledge of the biodiversity of the Sundaland that will help conservationists and natural resources managers in setting conservation priorities.

In the course of my dissertation research, I describe one new genus of bufonid toads—*Sigalegalephrynus* and five new species of this genus which are highly threatened and provisionally placed under the "Endangered" category of the IUCN Red List conservation

status (AmphibiaWeb 2020). I also discovered another new candidate species (sixth) of this genus which we also propose to place in the “Endangered” category of IUCN Red List status.

This study is the first and comprehensive molecular study on the genus *Phrynoidis* that uses samples from all of the major landmass of Southeast Asia. It clarifies the evolutionary relationships of Sumatran *Phrynoidis* populations and identifies the presently recognized Sumatran “*Phrynoidis juxtasper”* populations to represent an undescribed species.

Using molecular data and applying divergence analysis this study identifies five candidate species of *Leptophryne borbonica*. My study along with that of other researches of the Sundaland have contributed to a significant increase in species diversity in Indonesia (Fig. 1). This is a good example of using molecular data in taxonomic studies and finding biological units that will facilitate conservation in the region.

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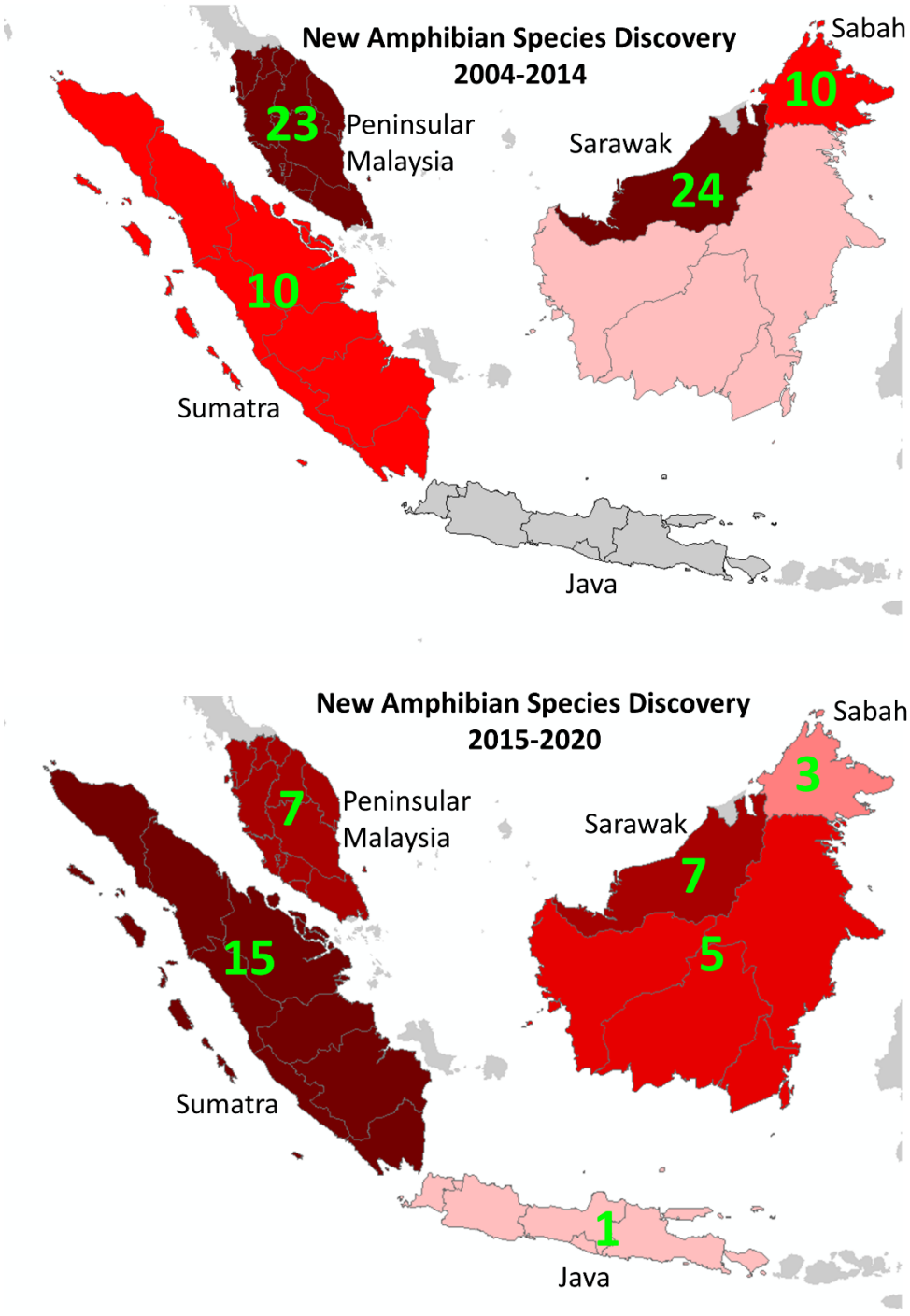

Figure 1. Comparison of new amphibian species discovery from major landmasses of the Sundaland from 2004 to 2014, and from 2015 to 2020.

BIOGRAPHY

Goutam C. Sarker was born at Chachitara Village under Manikganj District and Dhaka Division in Bangladesh. He graduated from Jahangirnagar University with a B.Sc. in Zoology in 2003. He obtained M.Sc. in Zoology with an emphasis in Wildlife Ecology, Management, and Conservation Biology in 2005 from the same institution. Goutam then joined Nature Conservation Management—one of the national pro-environmental organizations in Bangladesh—to the capacity of a Wildlife Biologist. In July 2006, he joined Dhaka City College as a Lecturer in Biology and was promoted to the rank of Assistant Professor in Biology in September 2011. He continued teaching at Dhaka City College until August 2013— before joinining the University of Texas at Arlington as a Ph.D. student. Goutam intends to continue his research on taxonomy, systematics, ecological niche modeling, and conservation biology of wildlife species to the capacity as a post-doctoral researcher or as a faculty member in a higher education institution.