DIVERSITY OF THE FANGED-FROG COMPLEX, *LIMNONECTES KUHLII* (ANURA: DICROGLOSSIDAE) ON THE MOUNTAINOUS REGIONS OF JAVA AND SUMATRA, INDONESIA

By

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DISSERTATION

Submitted in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF TEXAS AT ARLINGTON

DECEMBER 2021

Arlington, Texas

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Acknowledgments

Throughout the ups and downs of the graduate school journey in the Biology Department at the University of Texas at Arlington, I owe so much to so many people whose assistance and support would have made this process impossible. I would like to start with my advisor Dr. Eric Smith; his unwavering support and understanding of the nuances that come with being a graduate student are something I hope to take with me as I continue down my future path. His consistent mantra of, "… the way I see it, I would just be doing the same thing anyway." consistently echoes in my head as the way to live. I would also like to thank all of my doctoral committee members for challenging and guiding me through my dissertation research; Esther Betran, Todd Castoe, Matt Fujita, and Matt Walsh have all provided me a foundation to take forward into the wider world. I would especially like to thank Matt Fujita for allowing me to work with his lab through the COVID-19 pandemic, I would not be here if it were not for that assistance. In that same step the constant support of Kimberly Bowles at the Genomic Core Facility has also been immeasurable. The folks in the office who always made sure I registered, paid my bills, etc. including Gloria Burlingham, Ashley Priest, Linda Taylor, Chris Magno, Kathleen Demuth, and Mallory Roelke.

As all my fellow graduate students know we would not be anywhere without the mutual support of our peers. I would of course like to thank my lab mates first and foremost: Goutam Sarker, Justin Jacobs, Christian Hernandez among others. My good friends in the Passy lab; Will Budnick and Joe Mruzek for all the conversations both topical and not. TJ Firneno whose help and support cannot be measured on this or any other world. There are just so many people who even just a passing sentence has been helpful including: Brad Dimos, Andrew Corbin, Alex Murray, Rick Orton, Blair Perry, Danielle Rivera and just so many more. The countless ways in

which the graduate community can and should support one another I think any who have gone through the process can speak to.

For a while in the process, I was extremely worried about my ability to conduct the project I had outlined. The financial support I received from The Beta Chapter of Phi Sigma Society, Chicago Herpetological Society, Herpetologists Conservation International, and Society for Integrative and Comparative Biology for my research allowed me to go from 0 to 100 in the span of 3 months. COVID-19 then of course became the slowdown, but the support allowed me to think about "when" instead of "how." The support I received from the people in various herpetological organizations have also been incredibly helpful.

Most importantly my friends and family outside of academia. The consistent support from my parents and grandparents. They have never stopped believing in me and never will. The pick-me-up conversations to keep at it despite the difficulty constantly ring in my ears. My partner, Victoria Contreras, who has probably had to pick me up at my lowest points more than I would care to admit having.

To everyone I have mentioned and more, none of this would be possible without you.

Abstract

DIVERSITY OF THE FANGED-FROG COMPLEX, *LIMNONECTES KUHLII* (ANURA: DICROGLOSSIDAE) ON THE MOUNTAINOUS REGIONS OF JAVA AND SUMATRA, INDONESIA

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The cryptic species complex, *Limnonectes kuhlii*, previously was considered to have a large range across Southeast Asia. Recent works have started to identify species within the complex using primarily genetic methodologies. The largest absence in elucidating these relationships comes down to sampling. Traditionally sampling across Southeast Asia has been challenging, limiting sampling across the entirety of a species range. Nowhere is this more apparent than in Indonesia. Indonesia has been highlighted as a biogeographical hotspot for species despite limited sampling on any of the main or smaller islands across the archipelago. Countries such as Malaysia and Papua New Guinea that share borders with Indonesia on certain islands have much more sampling across a wider diversity of habitats and some such as the northern part of Borneo are even cited as areas of high speciation. This study uses the most extensive sampling across the islands of Java and Sumatra in modern reconning and utilizes those to look at genetic relationships across the islands. Utilizing sanger sequencing for parts of the mitochondrial genome and ddRAD-seq to sequence across the nuclear genome strong separation is identified between the islands of Java and Sumatra. Also utilizing several species delimitation methodologies 23 or 24 species are identified in both islands, currently hidden under the moniker of *Limnonectes kuhlii* with six clades being identified on Java and 18 on Sumatra. This study also identified clear morphological and morphometric differences between the skulls of males

and females of the *Limnonectes kuhlii* complex, highlighting overall head size as a clear difference between sexes. A study leading to a comparison between males of closely related species, and skull morphology being useful to identify differences in closely related species. Combined, all the methods used here, can assist in further identification of the species within the *Limnonectes kuhlii* complex on the islands of Java and Sumatra.

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Chapter 1: A Brief Introduction to Genus *Limnonectes* and the *Limnonectes kuhlii* Species Complex

Limnonectes Fitzinger, 1843 is a genus of frogs known more commonly as "fanged frogs," currently representing 81 known species across Southeast Asia ("AmphibiaWeb," 2021). Species in the genus are known for having high amounts of variation in reproductive modes, webbing, and size (Inger et al., 2017). Many species in the genus occur in sympatry, with mature forests of Northern Borneo containing three to five species (Inger et al., 2017). Most *Limnonectes* species frogs within the genus have noticeable odontoids on the lower jaw, hence "fangs." Most of those frogs are considered species of least concern and where they occur; however, populations are also listed as decreasing (van Dijk et al., 2004).

Limnonectes kuhlii is regarded as a species complex exhibiting highly conserved morphology. The main trait to define a species that belongs to the *L. kuhlii*-complex is that the tympanum is either hidden or partially hidden by the skin (Inger et al., 2017). They prefer rock streams with moderate currents and are never found far from them (Inger, 1966). The most recent systematic review of the *L. kuhlii*-complex denoted 22 distinct lineages, likely to be species (McLeod, 2010a). More would have been identified in the study had better sampling been applied. A further study of the Sumatran lineage by McLeod et al. (2011), with sampling limited to west-central Sumatra, described one new species over three distinct populations with one clade even suggesting a further species candidate. Other species have been uncovered within the complex, but Indonesia remained poorly sampled and with many species underrepresented (Figure 1.1 and 1.2).

Indonesia has been under-sampled compared to surrounding areas of Malaysia and the

Philippines, with the island of Sumatra being recognized as particularly poorly sampled (Inger

and Voris, 2001). While Indochina and Borneo have been highlighted as areas of high

diversification rates (de Bruyn et al., 2014), areas such as Java and Sumatra were not expected to

have a correspondingly high number of species due to a chaotic geologic history (Hall, 2012;

Inger and Voris, 2001). Compared to Sumatra, Java being a younger and smaller landmass would

be expected to have lower species diversity, as the Theory of Island Biogeography predicts

(MacArthur and Wilson, 1967).
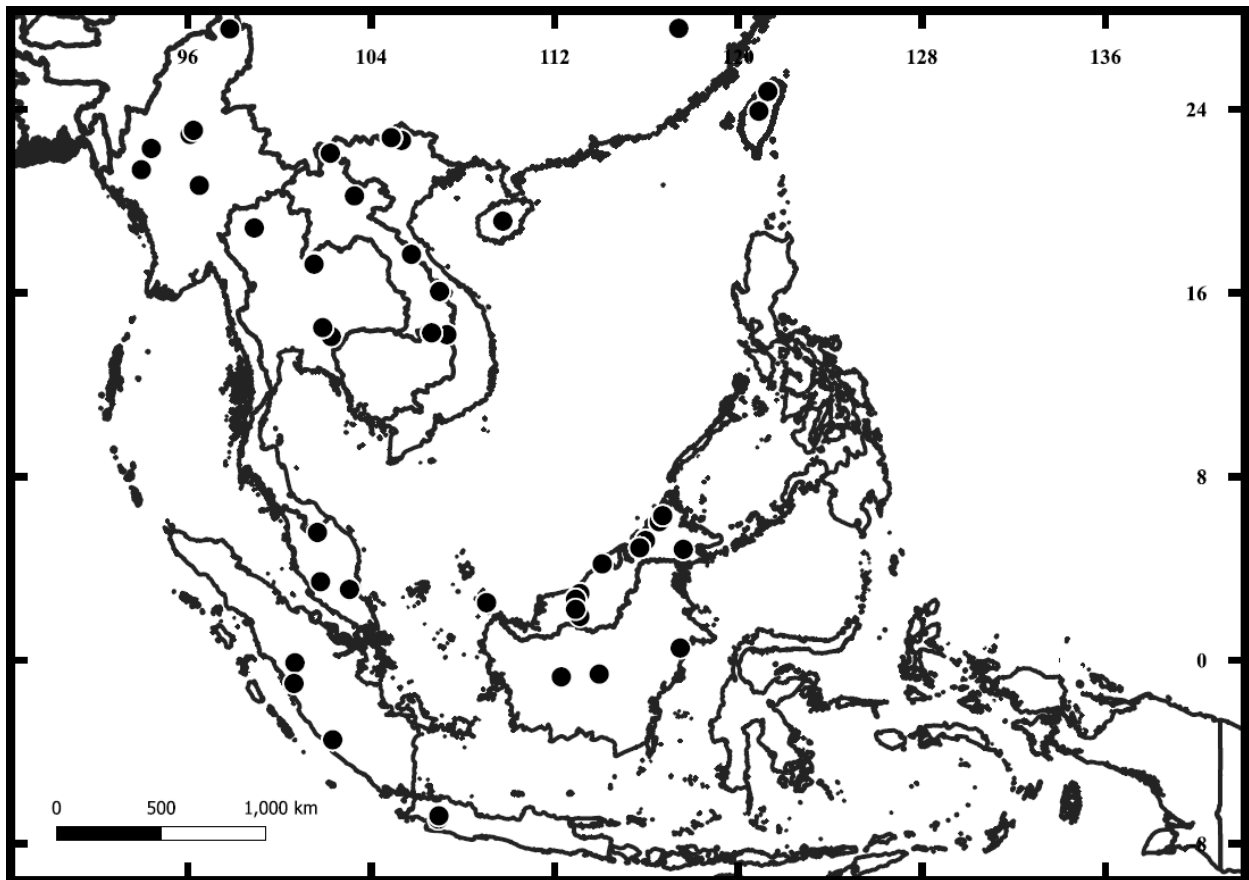
Figure 1.1. Map of Southeast Asia. Data points represent sampling from McLeod (2010a, 2011)

Taxonomy

The Genus *Limnonectes* Fitzinger 1843 currently contains 78 species (Frost, 2021). Since the McLeod (2010a) study, 23 species of *Limnonectes* have been described or re-established as species. Dubois (1986) separated much of what would be eventually be considered *Limnonectes* into several subgroups: *L. grunniens* (18), *L. kuhlii* (24), and *L. microdiscus* (10). One-third of *Limnonectes* lineages are considered members of the *L. kuhlii* group (Figure 1.2), based on morphological characters. Many species in the *L. kuhlii*-complex have been identified as species primarily through genetics, supported by some meristic characters (e.g.(McLeod et al., 2011; Siler et al., 2009). In addition to genetic evidence, biogeography assists in identifying and comparing similar species. The genus type *L. kuhlii* requires re-evaluation to determine which Javan population/lineage represents "true" *L. kuhlii*, a practice with implications not only for populations in Java but possibly also those in Southern Sumatra.

There might be at least two distinct evolutionary lineages within Java (McLeod, 2010a) and which lineage represents the "true" *L. kuhlii* informs awaiting taxonomic changes. Any idea of what "true" *L. kuhlii* is might be lost with specific records of Tschudi (1838), who is credited with naming the species. The syntypes at the Naturalis Biodiversity Center are data deficient, with the only useful information on the record being the locality of Java, for two samples in the series. Other designated types (lectotypes, paratypes) are met with the same locality designation of Java, Indonesia (Frost, 2021). The lack of more specific locality and descriptive information creates a problem in determining what "true" *L. kuhlii* is, as McLeod's (2010b) examination demonstrates differences between two of the considered holotypes from Leiden and Paris, respectively. The confusion of "true" *L. kuhlii* is as much a problem in its initial descriptions.
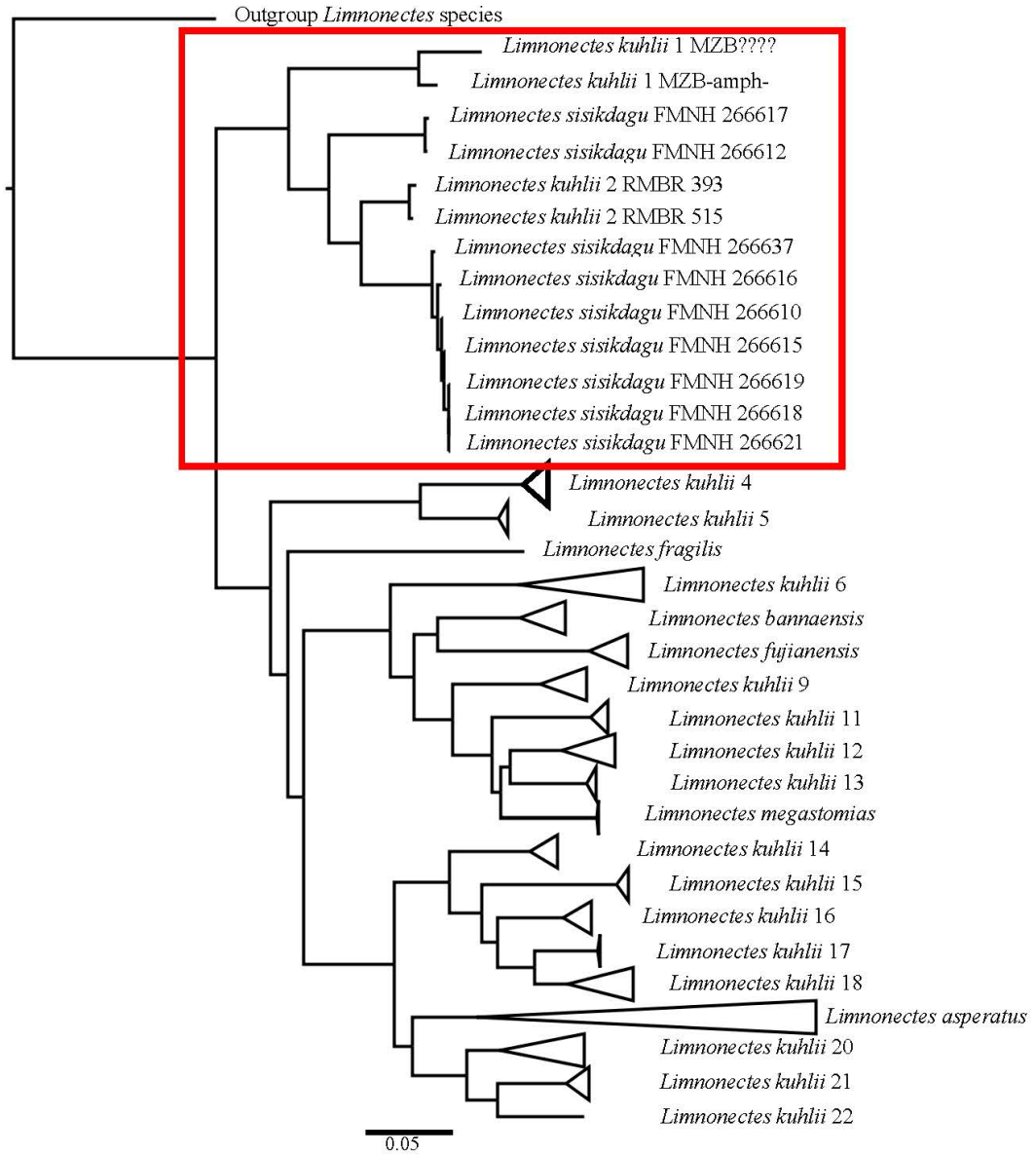
Figure 1.2. Tree represents *Limnonectes kuhli*i identified from McLeod (2010a, 2011). Red box highlights all current genetic sampling from Java and Sumatra prior to this work. *Limnonectes kuhlii* 1 represent species group from Java and *L. kuhlii* 2 and *L. sisikdagu* represent species groups previously identified on Sumatra.

Limnonectes kuhlii is named for the researcher Heinrich Kuhl, who made multiple discoveries in Java in the early 19th century. *Rana kuhlii* was initially differentiated from *R. macrodon* by Tschudi in 1838. Naming credit for this species is given to Tschudi (1838), where he describes *Rana kuhlii* as having a short, disheveled head with well-developed webbing (own translation). Limited description and poor locality information still plague identification for the widespread complex today, 180 years later, as their locality is listed only as "Java." Other synonyms include *Rana palmata* (Tschudi, 1838), *Nyctibatrachus sinensis* (Peters 1882), *Rana kuhlii* (Dubois 1981), *L. kuhlii* (Dubois, 1987; Fei et al., 1990). *Rana palmata* is introduced as a synonym to *R. kuhlii* within the same Tschudi (1838) work, which describes *R. kuhlii*. *Nyctibatrachus sinensis* was initially considered a synonym to *L. kuhlii*; however, since the description of *L. fujianensis* Ye and Fei 1994, *N. sinensis* has been designated as a synonym to *L. fujianensis* (Frost, 2021). Most of the more modern synonyms are a result of nomenclature changes from the 1980s into the 1990s, while the genus *Limnonectes* was being discussed and eventually widely accepted.

The current state of the *Limnonectes kuhlii*-complex comes from McLeod (2010a). He highlights 22 distinct lineages, most of which are either associated with mainland Southeast Asia or Malaysian Borneo, with only four clades from five localities across Indonesia in the study. McLeod et al. (2011) attempts to explore Sumatran species associated with the *Limnonectes kuhlii*-complex, as *Limnonectes sisikdagu*; a limitation of their study is only covering three regions of western Sumatra, all south of the Toba caldera, whose eruption likely reduced the species diversity dramatically in the area (Inger and Voris, 2001; Wilting et al., 2012). The three Sumatran populations of *L. sisikdagu* are all genetically distinct, and McLeod et al. (2011) highlight that they might represent different species, sister to Javan samples of *L. kuhlii*. This

evidence also shows a significant genetic distance for at least two Javan lineages of *L. kuhlii*. The Javan samples were collected at locations listed as "Java Island," "Java Barat Province: Kecamatan Kaduduampit," and "uncatalogued" in the study.

Life History

Limnonectes kuhlii are medium to large frogs distinguished from other *Limnonectes* by a partially hidden or completely hidden tympanum and having stocky limbs and fully webbed feet. Coloration in life is varying shades of brown to black dorsally and white ventrally, sometimes with spotting on the underside of legs (Inger et al., 2017). *Limnonectes kuhlii* are also known as "wart frogs," as parts of their skin are usually covered in tubercles. Differing patterns of tuberculation on the upper legs have been used as evidence to delimit species within the complex (McLeod et al., 2011).

All species within the *L. kuhlii*-complex are commonly associated with lotic environments, generally in hilly or mountainous forests, and never found more than a few meters away from a stream. The streams they associate with are clear water streams in both pristine and disturbed habitats, where they can be common. They are found between elevations of ~0 m to ~1800 m (Inger et al., 2017; van Dijk et al., 2004). Diets typically consist of invertebrates, such as coleopterans, diplopods, chilopods, orthopterans, and hymenopterans, with only one described account of a vertebrate (*Tropidophorus beccari*) found in stomach contents (Boon-Hee et al., 2010). Despite the larger head size in males, there is no functional difference in the diets between the sexes, though males have been reported preying on larger hymenopterans. However, this feeding difference is attributed to microhabitats associated with the sexes rather than sexually dimorphic feeding behavior (Emerson and Voris, 1992).

Common anuran secondary sex characteristics, such as nuptial pads, vocal sacs, and stocky forelimbs, are all diminished (e.g., nuptial pad of *L. sisikdagu*) or completely absent (e.g., nuptial pad of Javan *L. kuhlii*), with rare exceptions (e.g., vocal sac present *L. namiyei* [McLeod, 2010b]). Males are larger, with larger heads and longer odontoids or "fangs" than females. Increased head size primarily accounts for increased body size in males, as observed in Malaysian Borneo (Inger, 1966) and China (Pope, 1931).

The absence of vocal sacs was thought to be a key characteristic for several groups of fanged frogs (*Limnonectes kuhlii*, *L. blythii*, *L. ingeri*, and *L. ibanorum*) and, because of this feature, many are considered "voiceless" (Emerson and Barrigan, 1993). Though lacking vocal sacs, they are not truly voiceless as several males have been observed calling (Matsui, 1995; McLeod et al., 2012; Pope, 1931; Tsuji and Matsui, 2002). In *L. blythii*, another voiceless species associated with the *L. grunniens* group, females have been observed approaching male nesting sites and calling to males (Emerson, 1992). Tsuji and Matsui (2002) observed two different vocalizations: one warning call to both males and females for intruding upon the territory and another more aggressive call to intruding male frogs. While much of the behavior of *L. blythii* is typically associated with species in the *L. kuhlii* group, no female nest approaching behavior has been observed in species within the *L. kuhlii*-complex.

Males and females typically are found in different microhabitats. Males of *L. blythii* move between limited locations near the banks of a calm stream with either gravel bedded or some vegetation, which are considered typical of nesting sites for the species (Emerson and Inger, 1992). Males will defend their nesting sites from other males through male-male combat. Females are found most often in areas that would not be considered desirable for egg-laying. In the sites that are typical of females, males are never found. Females can also be found in the

nesting sites where they are observed mating (Emerson and Inger, 1992; Tsuji and Lue, 2000).

Control of the limited supply of nesting sites would be advantageous to the males that are able to

control them. Thus, male-male fighting has been thought to be the reason for the size

dimorphism between males and females (Emerson, 1992; Emerson and Inger, 1992; Emerson

and Voris, 1992; Shine, 1979). Fighting between *L. kuhlii*-complex males has been observed in

Taiwan (likely *L. fujianensis*), and in almost all observations the larger male was the victor

(Tsuji and Matsui, 2002) suggesting that the size of males equates to reproductive success

through control of territory.

Eggs are laid in calm stream sections near the banks. In the case of *Limnonectes kuhlii*

from Mount Kinabalu of Sabah, Malaysia (Northern Borneo), eggs are attached to bank and

stream vegetation (Malkmus et al., 2002). Other records have larvae found in calm pools

adjacent to swift streams (Inger, 1966). Tadpoles hatch after approximately ten days. While

tadpoles are found readily, their description and developmental morphology remain unknown for

L. kuhlii. Mouthparts are similar among all species within the complex, with few differences

among them throughout their range.

Biogeographical History of Two Major Islands on the Sunda shelf: Java and Sumatra, and that

History's Impact on the Islands' Biodiversity

The primary regions of focus for this study are the islands of the Sunda shelf, specifically the

montane regions of the two major islands of Java and Sumatra. Tropical regions, such as

Indonesia, house the highest levels of diversity of species (Böhm et al., 2013; Mittermeier et al.,

2011; Myers et al., 2000). Indonesia, in particular, is hyper-diverse, as it has tropical climate

regimes which traditionally house more species. This region's diversity can also be attributed to

global cooling and warming periods. The seas recede as glaciation occurs elsewhere, allowing the connection between the islands and the mainland and creating opportunities for dispersal. Conversely, warming events separate the islands. The geological history and historical climate patterns shape how species have been able to diversify across the Sunda region. Recently, however, human instigated habitat destruction and declines, such as climate change and habitat loss, threaten the rich biodiversity of this region.

Currently, the assemblage of the Sunda region is separated into five main regions: Borneo, Java, Sumatra, Malaysian Peninsula, and Indochina and the smaller Lesser Sundas. Islands pertinent to this study (Java and Sumatra) have a combined 11 ecoregions Wikramanayake et al., 2002). Java has four ecoregions based on the East/West axis: Java-Bali montane rainforest (eastern), Eastern Java-Bali rainforests, Western Java montane rainforests, and Western Java rainforests (Wikramanayake et al., 2002). Sumatra is larger and contains seven ecoregions: Mentawai Islands rain forests, Sumatran freshwater swamp forests, Sumatran lowland rain forests, Sumatran montane rain forests, Sumatran peat swamp forests, Sumatran tropical pine forests, and Sunda Shelf mangroves (Wikramanayake et al., 2002).

The climate of Indonesia is tropical, which is dictated more by the monsoonal periods than by temperatures. Temperatures are generally 28ºC at the coastal plains and 26ºC in lower mountain elevations, but reaching averages of 23ºC at higher elevation mountain ranges, with all months staying above 18ºC ("Climate of the World: Indonesia," 2018). The monsoon seasons are characterized by seasonality of heavy rains during the monsoon seasons and slight reduction in precipitation during dry seasons. Other events, such as cooler than normal sea surface temperatures resulting from El Niño, will also affect the amount of rain, particularly during the dry seasons with less notable effects in the wet season (Voris, 2000).

The primary Sunda region of Indonesia is known as the *Ring of Fire* because of the numerous volcanos that exist in the region, the most famous of which is Krakatoa on the Southern part of the island of Sumatra, which last erupted in 2020. While Krakatoa remains one of Indonesia's most famous volcanos, it is hardly the most influential to floral and faunal communities of Indonesia. The eruption of the mega-volcano, Toba, had a large influence on the climate and vegetation and, subsequently, the species assemblages on Sumatra (Wilting et al., 2012). Earthquakes and tsunamis have also shaped the Sunda region, with 49 significant earthquakes (criteria: 1 million+ damage, 10+ deaths, magnitude 7.5+, or generated tsunami),18 with associated tsunamis, since the year 1900 ("Global Significant Earthquake Database, 2150 B.C. to present," 2018). All of these natural events create opportunities for recolonization from previously occupying species, as well as new colonization events.

Throughout paleo history, the islands of the Sunda shelf have cycled between highly connected and highly isolated. These connections and separation events act on the ability of species movements: connections allowing species to colonize the other islands in the region, and the separations providing periods of isolation leading toward the possibility of diversification. During the Holocene, the Earth oscillated between periods of glaciation and warmth. Many periods of connectivity and separation occurred between what is now Java and Sumatra and the Malaysian Peninsula (Barber et al., 2005).

The geologic history of Sumatra has been complex since its primary separation from the Malaysian Peninsula in the Earliest Miocene (Barber et al., 2005; Hall et al., 2009; Meijaard, 2004). The primary landmass of Sumatra was submerged with three main volcanic islands. In the Late Miocene, the tectonic plates below Sumatra and Borneo rotated the islands into their present-day positions; an increase in uplifting events and volcanic activity began, which

continues through today throughout the *Ring of Fire* (Barber et al., 2005). These ongoing uplifting events would create mountain ranges that would isolate species as they adapted to the changing elevation. Once the island of Sumatra was mostly above sea by the Early Pliocene, many of the species would have been already isolated on various highland regions.

The island of Java is not as complex as Sumatra, with west Java having a significant landmass in the middle to early Late Miocene (~10mya) (Meijaard, 2004). Parts of east Java would emerge in several volcanic clusters toward the end of the Miocene with continued uplifting events and volcanic activity from the tectonic plate rotation (Barber et al., 2005; Meijaard, 2004). The early to middle Pliocene would have Java in three main parts: a western island, a central island, and an eastern island, with the Middle Pliocene separating further from both eastern and western islands for a bit. The Late Pliocene sees the development of highlands in central Java, with highlands finally connecting all of Java in Early-Middle Pleistocene (Meijaard, 2004).

The most recent glacial maxima began to recede around 17000 years ago. At that time, sea levels in the equatorial region of Southeast Asia were approximately 120 m below current levels (Voris, 2000). At the lowest level, all of the Sundas were connected to the greater Asian continent, allowing for species to colonize both ways. Dispersion would have been less likely for this study as this work focuses on highland species. The islands became isolated from peninsular Malaysia when the sea level increased; Borneo separates when the sea is 30 m below present levels, and Java and Sumatra separate, both from the mainland and each other when the sea level reaches 20 m below present levels (Voris, 2000). Free movement across peninsular Malaysia, Sumatra, and Java would effectively end around 8000 years ago. Borneo and Indochina have seen higher levels of emigration during cooling periods (de Bruyn et al., 2014). The cooling

periods throughout the various epochs highlight Borneo and Indochina as areas with higher levels of diversification (de Bruyn et al., 2014), which would account, in part, for the high number of *Limnonectes* species associated with the *Limnonectes kuhlii*-complex currently recognized in Borneo and Indochina compared to other regions in the Sunda shelf.

Geologic stability observed in Indochina and Borneo could have driven higher levels of diversification, compared to places such as Sumatra and Java (Inger and Voris, 2001), which are both younger islands and were subject to uplifting events starting in the Late Miocene (Barber et al., 2005; Barber and Crow, 2005; Hall et al., 2009). The climate during the cooling periods is thought to have transformed evergreen rainforests to grassy savannahs, possibly containing some gallery forests, which would have created dispersal paths for species restricted to these environments (Voris, 2000). Alternatively, some studies suggest the existence of rainforest refugia in various spots of the Sunda shelf, particularly Mentawai Islands, Northern and Eastern Borneo, and Northern and Western Sumatra (Gathorne-Hardy et al., 2002). Other studies suggest the rainforests and the savannahs were more extensive at the last glacial maxima, and thus today's forests are the refugia (Cannon et al., 2009; Woodruff, 2010). Both of these ideas on refugia could have implications on the speciation of the *Limnonectes kuhlii*-complex regarding both the concept of recent isolation and the recolonization from hypothesized forest refugia.

Volcanos have shown to be a large influence on the islands of Sumatra and Java. Most of the peaks in these areas of the Sunda shelf are volcanic and have affected much of the land, as the subducting regions that form the Sumatra and Java trenches increased the mantle temperature of the region, thus increasing volcanic events in the Eocene (Hall, 2012). As the Australian plate moved north, the subducting Australian plate around the Java trench was particularly active. The subduction can be constructive, with orogenic activity creating new montane habitats at higher

elevations to be colonized. However, tectonic activity can also have a destructive influence on communities. The eruption of the Toba super-volcano in central Sumatra caused a temporary global cooling with estimates of 5–15 C below normal (Jones, 2007). Global cooling would create droughts in areas with more monsoonal rainfalls. Anuran species such as *Ansonia* are mainly dependent upon monsoonal rainfall (Inger and Voris, 2001). Wilting et al. (2012) suggest the Toba eruption and the resulting climate change are not fully able to be separated from one another as the cause for the extinction events. These events are considered the likely reason for many mammal species on the West Sumatran Island of Mentawai being more closely related to Borneo than Sumatra. In contrast, amphibians do not seem to follow this same pattern (Wilting et al., 2012).

Human influences have significantly affected the environments of the islands of Indonesia. Sumatra has some of the highest deforestation rates in Indonesia (Hansen et al., 2009) and primary forest, even in montane regions with difficult access, is seeing higher rates of deforestation (Margono et al., 2014). One of the more significant threats to forested areas in Indonesia is palm oil, though coffee and latex plantations also threaten the remaining forest. As a crop, palm oil is generally a monoculture that requires very humid environments to grow effectively, which is one reason that Indonesia has come to produce much of the world's palm oil. While the cultivation of palm oil is slowing in Malaysia, the palm oil industry in Indonesia is expected to continue growing as demand continues to rise (FAO, 2012).

One of the problems associated with land use in Indonesia is the inaccuracy of maps. The maps are often inconsistent with each other, and owning rights can be contested (Cronkleton et al., 2008); this can sometimes lead to one person or organization making a claim to go and develop the land that may not even be theirs. The Indonesian government does have a plan to

combat this, which is the One Map Initiative (*Indonesia - Program to Accelerate Agrarian Reform (One Map Project) (English)*, 2018), an initiative that the Indonesian government hopes will quell land disputes and that the companies will set aside prescribed natural areas. Other claims, such as traditional tribal forests under previous laws, may also lead to a more locally regulated forest under village ownership.

Indonesia is home to some impressive wildlife, including Sumatran tigers, Sumatran Rhinos, and water monitors. This wildlife is attractive to poachers, who illegally collect and export many species. Some species, such as Sumatran Rhino, are taken for Chinese medicine (Challender and MacMillan, 2014), while others, such as *Lanthanotus borneensis* (earless monitors), are considered attractive to collectors in the pet trade. The poaching on the Indonesian wildlife is a large cause for concern as Indonesia remains largely poorly explored and, as a result, many species remain undescribed; recent estimates state that 33% of amphibian species remain to be identified (Giam et al., 2012). While more measures have been put in place to deter the issue, lack of enforcement remains a significant problem for much of Indonesia.

Indonesia's population growth has created high demand for land, driving people into more sensitive forest areas. As the fourth most populous country in the world, with an annual growth rate of 1.58 (*Indonesia - Program to Accelerate Agrarian Reform [One Map Projec] [English]*, 2018), people find themselves in need of property for homes, farms, etc. In Indonesia, indigenous tribes are relatively unprotected regarding property rights; as more migrants press into these lands on the tails of productive operations, such as commercial logging, the indigenous people are forced further up the mountain (Poffenberger, 2006). Government management of forests of Indonesia has been very inconsistent over the years, traditionally favoring large conglomerates or creating vague rules of protection for management (Jepson et al., 2001).

Presently, Indonesia does not have much land set aside for conservation, with only 14% set aside for preservation across the entire country (Shah and Baylis, 2015). Ecotourism is being explored as a mechanism to preserve more land, though presently, it does not appear to mitigate conservation threats for many species of concern. However, proponents of ecotourism point out that some species are seen to maintain or even improve with varying levels of ecotourism (Buckley et al., 2016). With a better understanding of species and their boundaries across the archipelago, decisions regarding species conservation might be made more effectively.

This study uses the largest collection of specimens across Java and Sumatra for phylogenetic sampling. Molecular data generated for this study includes methods in Sanger sequencing as well as double-digest restriction-site associated DNA (ddRAD-seq), including sequences from the online databank, GenBank. This study provides a look at the phylogeny and phylogeography of a cryptic species complex within the confines of a traditionally poorly sampled landscape. For my dissertation research, I used morphological, molecular (mitochondrial DNA, ddRADs), and distributional/geospatial data to highlight populations and identify current distributions through environmental niche modeling.

My second chapter utilizes fragments of 12S and 16S rRNA genes and SNPs generated from ddRAD-seq to explore molecular systematic relationships among populations of species within the *Limnonectes kuhlii* complex on the islands of Java and Sumatra. This study aims to expand upon Indonesian lineages included by McLeod (2010a) and McLeod et al. (2011). The split between Java and Sumatran species is well supported. Each island also has one large, highly supported division. They differ in that the divisions on Java are supported by geography, while Sumatra appears to be much more complicated. Species delimitation methodologies suggest many species that will need further evaluation.

In my third chapter, I explore how Java and Sumatra are both relatively young islands, with modern full connectivity of Sumatra arising in the Late Miocene with a coupling of volcanic and tectonic events (Barber et al., 2005) make the biogeographical history of species within the *Limnonectes kuhlii* complex worth exploring. The split between Sumatran and Javan *L. kuhlii* coincides with West Java's formation in the Mid-Late Miocene.

The fourth chapter explores morphological variation both within and between species in the *Limnonectes kuhlii* complex. Sexual dimorphism is common across many amphibian species. In species within the *L. kuhlii* species complex, the males are larger than the females, primarily due to the size of the male head (Inger, 1966; Pope, 1931). While no direct observation has been made regarding behavior within the species complex, species in the closely related *L. grunniens* group display similar behavior with females approaching male nesting sites (Emerson, 1992). Male-male combat has been observed in closely related species of *Limnonectes,* with larger males typically being the winner (Tsuji and Matsui, 2002), and males and females are often found in separate microhabitat (Emerson and Inger, 1992). This intraspecific competition would lead to pronounced traits within males, whereas females would not be faced with the same morphological selective pressure. Males display higher amounts of divergence between closely related species, whereas females display less if any morphological variation.

Chapter 2: Phylogenetics of *Limnonectes kuhlii* (Anura: Dicroglossidae) on the Islands of Java and Sumatra, Indonesia

Introduction

The genus *Limnonectes* currently contains 81 species of frogs and counting, with four new species described in 2021 alone ("AmphibiaWeb," 2021). Species within the genus are known for variation in reproductive modes, webbing, and size (Inger et al., 2017; Setiadi et al., 2011), with many species appearing in sympatry with others within the genus (Inger et al., 2017). The type species of genus *Limnonectes* is *L. kuhlii* Tschudi (1838) and, though once considered extremely widespread across Southeast Asia, is recently viewed to have a much more restricted distribution within the island of Java (McLeod et al., 2011). The *L. kuhlii* complex currently represents over 22 distinct lineages across Southeast Asia (McLeod, 2010a), with only *L. kuhlii* and *L. sisikdagu* as recognized species on the Indonesian islands of Java and Sumatra, respectively (McLeod et al., 2011). Two primary reasons exist for the lack of diversity within Indonesia, highly conserved morphology within the species complex and a lack of collections from across the region with even fewer modern collections containing tissue samples that can be used for genetic identification.

Species within the *L. kuhlii* complex are nearly impossible to tease apart through simple morphological means and are thus considered cryptic species. Cryptic species arise in three main ways: 1) they are young species, 2) niche evolution, and 3) morphological convergence (Fišer et al., 2018). As a result, they can remain hidden in areas of hyper-diversity. The fanged frog species complex, *Limnonectes kuhlii*, is lineage rich (Matsui et al., 2016) with many undescribed species due to limited sampling, despite genetic evidence suggesting that additional lineages are

present throughout Southeast Asia (Evans et al., 2003; Matsui et al., 2016; McLeod, 2010b, 2010a; McLeod et al., 2011). By improving the sampling coverage across the *L. kuhlii* range and using genetic tools to uncover the diversity within its range, this species complex can provide insights into mechanisms of speciation in hyper-diverse regions.

Young species can arise due to recent allopatric separation, which may have recently occurred in the Sunda shelf as geologically recent variations in connectivity have occurred between the islands and the mainland, possibly separating populations (Voris, 2000). Niche evolution can occur when new niches form due to the geological volatility, which is characteristic of the Sunda shelf (Hall, 2012; Wilting et al., 2012), leading to novel selective pressures. Morphologic convergence can arise through convergent evolution of the same physical trait obscuring patterns of evolution and leading to species misidentification.

Several Islands of Indonesia have been recognized as being poorly sampled due to many different situations, including access to environments (von Rintelen et al., 2017). Despite the limited sampling, some regions of Indonesia have shown significant differences in diversification rates. Some factors influencing the diversification rates range from their diverse geological histories, a wide variety of island sizes and ages, and sea-level fluctuations leading to periods of connectivity conducive for dispersal and vicariance events. While Indochina and Borneo have been highlighted as places of high diversification rates (de Bruyn et al., 2014), the chaotic geological history of Sumatra is thought to limit the number of species present on the island (Inger and Voris, 2001). Java and Sumatra have chaotic geological histories and are relatively young landmasses, compared to other islands within the Sunda Shelf, such as large parts of Borneo (Hall, 2012). Additionally, according to the Theory of Island Biogeography, Java would also have a limited number of species due to its smaller size (MacArthur and Wilson, 1967).

Such varieties in island size, age, and geological histories have resulted in Indonesia having vastly different diversification rates throughout the country, with regions such as Sumatra and Java providing comparably limited diversity for sampling.

Herein, we utilize ddRADseq data to uncover lineages within the *Limnonectes kuhlii* complex on the islands of Java and Sumatra. It is expected that multiple populations will be uncovered within *L. kuhlii* and *L. sisikdagu,* revealing populations with fairly resolved admixture that are associated to primary geographic ranges. Utilizing gene-tree analyses, we seek to answer the following questions: (1) Do populations within islands form monophyletic groups? (2) How does population structure look like for *L. kuhlii* on the islands of Java and Sumatra? (3) Do populations show a lack of gene flow between populations, suggesting unidentified species?

Materials and Methods

Taxon Sampling and DNA Extraction

Our taxon sampling consisted of 67 individuals across Indonesia (Figure 2.1). We collected and acquired tissue from them between the years of 2012 and 2016. We extracted DNA from muscle or liver tissue stored in 1.5 mL of cell lysis buffer solution (0.5 M Tris/0.25% EDTA/2.5% SDS, pH = 8.2) using a phenol-chloroform protocol (Green and Sambrook, 2017; Sambrook and Russell, 2006). We checked the quality of our DNA extractions using a 1% agarose gel and quantified the DNA concentrations using an AccuGreen ™ Broad Range DNA kit (Biotium, Inc, Fremont, CA) on a QUBIT 2.0 Fluorometer (Life Technologies, Carlsbad, CA).

We collected ddRADseq data for 67 individuals associated with the *Limnonectes kuhlii* complex (Figure 2.2 and Appendix 1) following the protocol described in Peterson et al. (2012)

and following parameters specified in Streicher et al. (2014). The double digests were conducted using 500 ng of DNA per individual using 20 units each of SbfI and MspI and digested for 5 hr at 37°C in 1x Cutsmart Buffer (NEB). Barcoded Illumina TruSeq adapters were ligated at 23°C for 30 min and then heat kill the enzyme for 10 min at 65°C. Adaptors included an 8-bp unique molecular identifier (UMI) to reduce poor quality sequence ends. Up to 12 UMIs were pooled into a group, each with a TruSeq single index. The RAD library product was checked on a test gel of 1.5% agarose. We size selected nine groups using the Blue Pippin electrophoresis platform (Sage Science, Beverly, MA, USA) for 435-535 bp fragments. The RAD PCR libraries were amplified using indexed Illumina paired end PCR primers using Phusion High Fidelity Proofreading Taq on a thermocycler for 30 s at 98°C followed by 15-20 cycles of 30 s at 98°C, annealing for 30 s at 55°C, 1 min at 72°C and 5 min at 72°C with a final rest at 12°C. Successful libraries were confirmed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using a DNA 7500 chip kit. Final concentrations were verified using the Qubit 2.0. We sequenced our final pooled ddRADseq library on one Illumina NovaSeq S4 flow cell lane (150 bp single end reads) through the company Novogene (Sacramento, CA).

We automated the workflow for data processing, filtering, and formatting using scripts available from Portik et al. (2017: https://github.com/dportik/Stacks_pipeline). In brief, we demultiplexed the raw Illumina reads using STACKS v1.35 (Catchen et al., 2013), removed the restriction site overhangs using the fastx_trimmer module of the FASTX-TOOLKIT (www.hannonlab.cshl.edu/fastx_toolkit), and examined the sequencing quality on a per-sample basis using FASTQC v0.10.1 (www.bioinformatics.babraham.ac.uk/projects/fastqc). We created, cataloged, and identified loci using USTACKS, CSTACKS, and SSTACKS, respectively. We then used POPULATIONS to generate three separate datasets: (1) a composite dataset of *L. macrodon* and *L.*

kuhlii samples to generate alleles for loci present in 70% of all individuals, which resulted in 621 loci, then removing "blank" loci (n = 0), invariant loci (n = 220), non-biallelic loci (n = 0), loci containing at least one individual with more than two alleles (n = 0), and samples with missing data for more than 50% of loci; (2) a dataset with only Sumatran *L. sisikdagu* samples to generate alleles for loci present in a minimum 70% of all individuals, which resulted in 889 loci, then removing "blank" loci (n = 0), invariant loci (n = 410), non-biallelic loci (n = 0), loci containing at least one individual with more than two alleles (n = 0), and samples with missing data for more than 50% of loci; and (3) a dataset with only Javan *L. kuhlii* samples to generate alleles for loci present in 80% of all individuals, which resulted in 3,268 loci, then removing "blank" loci (n = 18), invariant loci (n = 1,197), non-biallelic loci (n = 0), loci containing at least one individual with more than two alleles (n = 0), and samples with missing data for more than 40% of loci. After completing the above filtering steps, our final SNP datasets consisted of: (1) 67 samples and 401 loci; (2) 37 samples and 479 loci; and (3) 26 samples and 2,053 loci, respectively.

Phylogenetic-tree Analysis

Once we generated and processed the molecular ddRADseq data, we estimated phylogenetic trees using maximum likelihood (ML) and Bayesian inference (BI) criteria for the concatenated dataset. We used the program RAxML v8.2.12 (Stamatakis, 2014) on the CIPRES Science Gateway (Miller et al., 2010) with the GTR +I +G model for ML analyses with a random starting tree and all parameters estimated. We estimated the clade support values inferred by ML analyses with the rapid bootstrap algorithm with 1000 replicates (Stamatakis et al., 2008). We determine BI by running BEAST 2.6.3 (Bouckaert et al., 2019; Drummond and Rambaut, 2007;

Suchard and Rambaut, 2009) with 20,000,000 generations, sampling every 1000 generations. This resulted in the production of a total of 20,000 trees on the CIPRES Science Gateway (Miller et al., 2010). We then assessed the runs using TRACER v1.7 (Rambaut et al., 2018) to examine convergence. We discarded a burn-in of 10% and created a maximum clade credibility (MCC) tree with median heights from the remaining 18,000 trees.
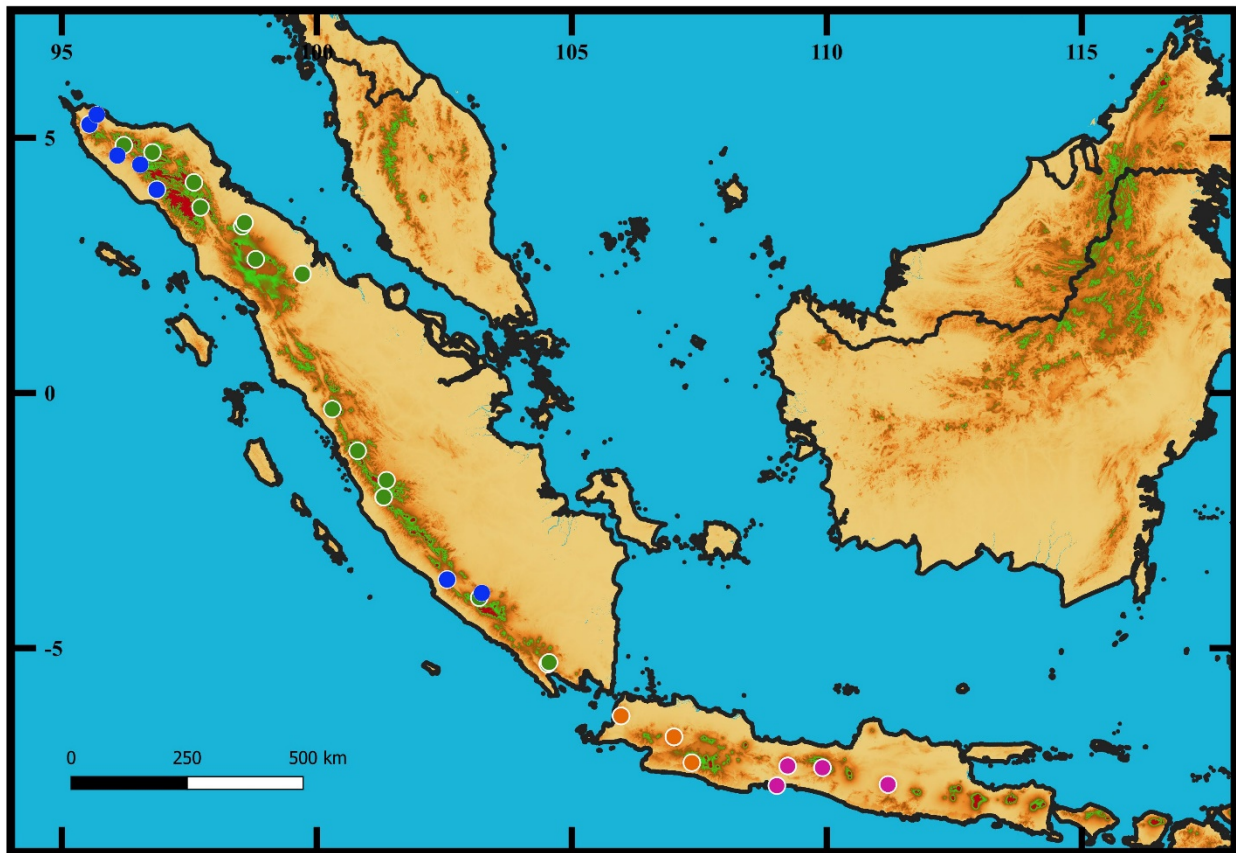
Figure 2.1. Sunda region of Indonesia with samples selected for ddRAD-seq. Blue and green dots represent the two major divisions within *Limnonectes sisikdagu* and orange and pink dots represent species from recent expeditions classified as *L. kuhlii*.

Population Discovery

We determined the number of discrete populations present across the sampled range of this complex using a maximum likelihood approach with ADMIXTURE (Alexander et al., 2009). We

performed ten replicate analyses to evaluate up to 30 populations. To assess the best value of k, we performed 10-fold cross-validation and determined the K value with the lowest cross-validation error. To independently assess the validity of our ADMIXTURE results, we used the FINERADSTRUCTURE software package (Malinsky et al., 2018) to construct a co-ancestry matrix from our RADseq data. We used a 100,000 burn-in followed by 100,000 MCMC steps sampling every 1,000 steps, and the tree was constructed with 10,000 hill-climbing iterations. We visualized the results using the FINERADSTRUCTUREPLOT.R and FINESTRUCTURELIBRARY.R scripts (included in the fineRADstructure package file).

Results

Phylogenetic-tree Analyses

Topologies for both ML and BI analyses were identical for ddRADseq datasets (Figure 2.2). For these analyses the *Limnonectes kuhlii* species complex formed a monophyletic clade, separate from the outgroups comprised of *L. microdiscus* and *L. macrodon*. The results included several well supported clades including major splits between Java and Sumatra. The Javan clade was further supported to have and east/central clade and a west clade. The west clade had support to separate into three well supported clades and the east had modest support for three clades within the sampling of east Java (Blue clade, Figure 2.2). Sumatra has two main highly supported clades, both of which are distributed across Sumatra. The clade for the first Sumatran group has four well supported splits that identify five clades (Yellow clade, Figure 2.2). The other well supported Sumatran clade contains several well supported clades all of which correlate to geography of the region (Red clade, Figure 2.2). The only exception for all samples cladding

within their respective islands is sample ENS 15097 from Banten, Java, which clades with

samples from the southern part of the Bengkulu Province in Sumatra.
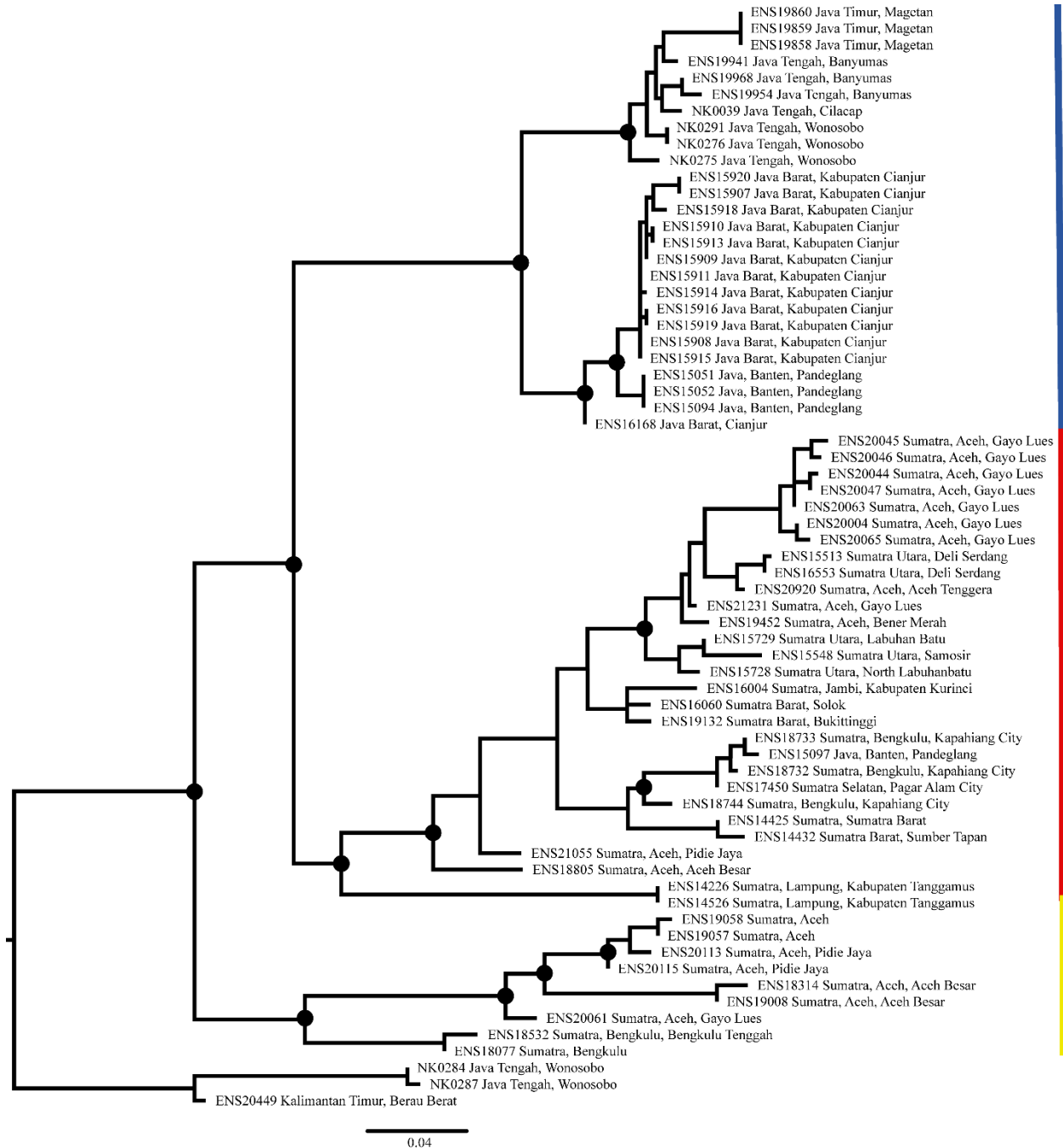
Figure 2.2. Maximum likelihood for ddRADseq data. Filled black dots represent high support for maximum-likelihood (> 90 support values). Blue represents clade from Java. Red and Yellow are the two major clades from Sumatra.

Population Discovery

The maximum-likelihood population clustering analysis based on 401 unlinked SNPs sampled across both Java and Sumatra for the *Limnonectes kuhlii* complex contained 25 populations (Figure 2.3, Figure 2.4). The cross-validation for k populations suggested that a k = 25 to be the best supported for all populations across Java and Sumatra. When removing the samples of *L. macrodon* then 24 populations across Java and Sumatra were resolved. The FRS plots showed similar trends of admixture, with most samples relating to one another based on relative geography (Figure 2.3). Admixture gave four populations for the "Yellow" clade (Figure 2.2) from Sumatra, all well resolved, with little admixture within populations (Figure. 2.3). The population structure for the "Red" clade from Sumatra gave 11 populations, most fairly resolved with some admixture. The Javan clade was suggested to have nine populations. The data suggests that East and West Java are genetically distinct from one another. East Javan samples showed three fairly resolved populations (Figure 2.3). West Java showed six populations, two well resolved, and four with lots of admixture. The samples of high admixture all are listed from the same locality in Java Barat, Kabupaten Cianjur, a mountain nearby to Bogor (Figure 2.4). The FRS plots were rerun for just Sumatra and just Java, respectively, and demonstrated the same trends as the admixture plot and the combined FRS plot. Clear support for two major clades in Sumatra with support for smaller clades based on geography, and Java showing two major clades, one for east, one for west, with smaller clades based on geographic location.
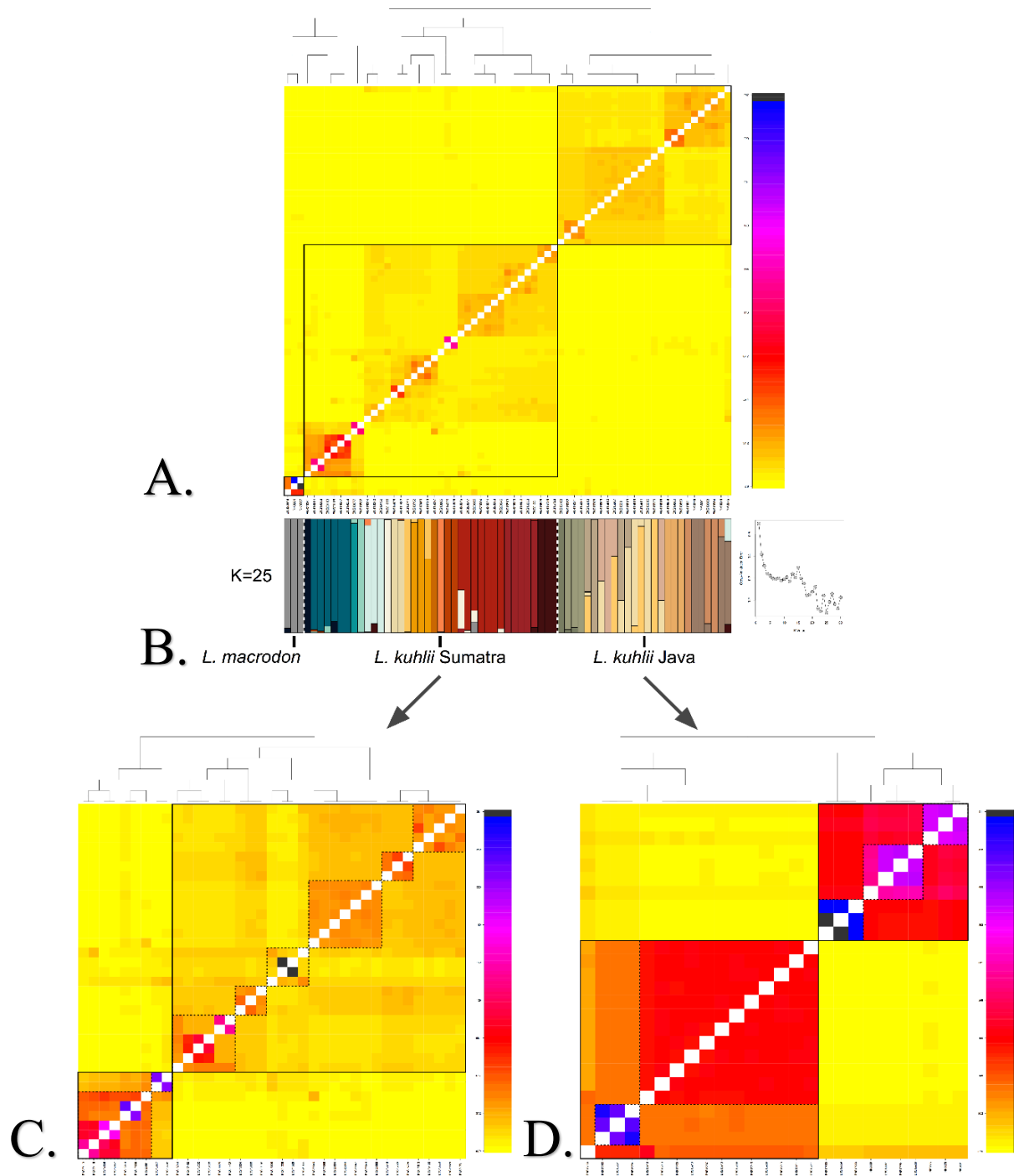
Figure 2.3. Fine RAD structure plots for combined Java and Sumatra (A), associated to corresponding admixture plot (B) based on DDRADseq libraries. Only Sumatra FRS plot (C) and only Jave (D), help to demonstrate the structure within islands more effectively. Heat maps demonstrate relatedness of the samples to one another; black represents extremely closely related, yellow distantly related. Colors from admixture plot correspond to colors used in pie graphs in Figure 2.4.
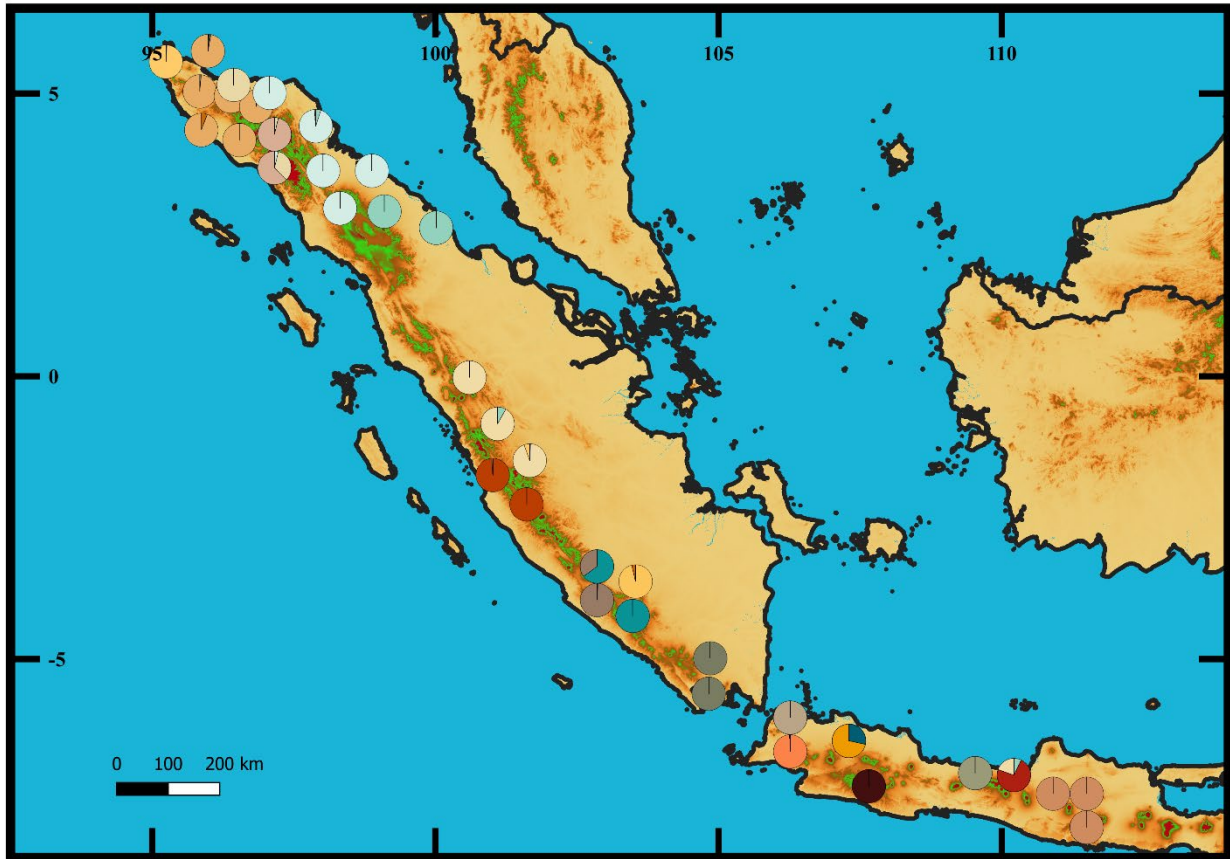
Figure 2.4. Map of samples across Indonesia from admixture plot for the best fit k = 25 across both Sumatra and Java. Colors correspond to the colors in the admixture plot in Figure 2.3B.

Discussion

This study uses ddRADs across part of the range for the *Limnonectes kuhlii* complex. We tested the population methodologies, treating the populations as one species across the islands, as well as looking at them on each island respectively. Based on all analyses, the diversity is considerably higher on both Java and Sumatra than previous studies have been able to evaluate.

The first major division in the *Limnonectes kuhlii* group suggests one Sumatran clade is sister to the clade that contains *L. sisikdagu* (Sumatra) and *L. kuhlii* (Java). The current classification (McLeod et al., 2011) supports all clades within the *L. kuhlii* species complex in

Sumatra to be classified as *L. sisikdagu*. The current phylogenetic placement of *L. kuhlii* is within *L. sisikdagu* (Figure 2.2). Surprisingly, Java seems to be mostly overlooked when species groups are phylogenetically explored. Most studies, may include samples from west Java, but will focus on limited parts of Sumatra, Borneo and the Malay Peninsula as an attempt to look at the whole of the Sunda Shelf (Chan et al., 2020; Inger et al., 2009; Lim et al., 2020).

Initial divisions between Java and Sumatra show that the clades generally are only found on either only Java or Sumatra. There is evidence that a southern clade of Sumatra (Bengkulu) and western clade of Java (Banten) share species. The sample ENS 15097 from Banten is found within a clade of samples from Bengkulu (Figure 2.2, Figure 2.4). Evidence of other Anurans having populations extending from eastern Java and into at least southern Sumatra such as *Duttaphrynus melanostictus* (Sarker et al., 2019) and *Chalcorana chalconota* (Inger et al., 2009; as *Rana*). However, those other species are generally found at lower elevations and would have seen possible exchanges during lower sea levels at the last glacial maxima (Voris, 2000), though the current separation between Java and Sumatra is considered to be a strong barrier to geneflow (Inger et al., 2009). Highland species such as the ones used in this study would be less likely to have dispersed at these times. Other support for populations occurring on southern Sumatra and western Java is the geology in common, more than between west Java does and central and eastern Java (Schlüter et al., 2002).

Populations

The population discovery had a k = 25 as the best fit number of putative populations when tested for one to 30 populations. The outgroup *Limnonectes macrodon* was also removed, which shared no genetic information with any of the ingroup populations. The main divisions seen from

maximum-likelihood support (Figure 2.2) were also scene in the FRS plots (Figure 2.3A) and

Admixture plot (Figure 2.3B). The FRS plots demonstrated two closely related clades on

Sumatra (Figure 2.3C) and two on (Java Figure 2.3D). Many of the groups were highly resolved

and have no geneflow between, suggesting presence of species and not only populations. Since

the species we are observing for this study are from highlands many populations diverge

genetically within specific mountains or mountain ranges (Camacho-Alpízar et al., 2018).

Uplifting events such as those forming mountains are known to initiate lineage divergence

(Shahzad et al., 2017), accounting for the genetic differences seen in the *L. kuhlii* complex of

Java and Sumatra. Some of the clades in North Sumatra show a wider range, but most are

restricted to small areas. Effectively movement would be restricted in species and populations

because of higher elevations and the elevation differential to disperse to other highland

environments (Ghalambor et al., 2006), even in times of island connectivity.

Java has the east-west division well supported with no admixture occurring between the

two larger clades (Figure 2.3D). Other groups have similar Javan relationships, showing an east-

west division sister to Sumatran clades (Cumming et al., 2020; Hudjashov et al., 2017). While

most of the populations were well resolved, four populations, all from Kabupaten Cianjur, in the

province of Java Barat, demonstrate high levels of admixture. These can often be interpreted by

effects of recent genetic drift or complex patterns from ancestral structure (Lawson et al., 2018).

The admixture also suggests the possibility of multiple introductions over time (Verhoeven et al.,

2011) which may be explained by the intense volcanic history of Java creating refugia (Garjito et

al., 2019) or areas that were colonized multiple times by slightly divergent populations.

The Banten sample embeded within a Sumatra clade in the maximum likelihood tree

(Figure 2.2) also associated with Sumatran samples in the k = 25 admixture plot (Figure 2.3 B)

and the FRS plot for Java and Sumatra (Figure 2.3 A) combined. However, when it was separated from the Sumatran clades and placed into the Javan FRS plot (Figure 2.3 D) instead of forming an individual group in the FRS plot it was placed within the clade with samples from Kabupaten Cianjur. The heat map supports this placement because of the amount of variance we see in the clade supported by the admixture results or ancestral phylogenetic signaling (Lawson et al., 2018).

Most of the populations as previously stated were well resolved in the admixture plot (Figure 2.3B). The heat maps from the FRS plots (Figure 2.3 A,C,D) further support strong structuring toward geographic populations. The genetic isolation of these populations suggests a deeper look into their relationships as species. In tropical habitats montane habitats often contain rich amounts of biodiversity (Sodhi et al., 2007) so the geographic support and genetic isolation seen from the populations in the *Limnonectes kuhlii* complex are not out of line. More lineages are still likely to be uncovered with more data, but these contributions start to emphasize the amount of diversity that remains hidden in Java and Sumatra.

Chapter 3: Biogeography of *Limnonectes kuhlii* (Anura: Dicroglossidae) on the Islands of Java and Sumatra, Indonesia

Introduction

Indonesia has a complex geological history with many periods of connectivity leading to episodes of isolation and dispersal (Barber et al., 2005; Hall, 2013; Meijaard, 2004). The islands within the Sunda Shelf (Sumatra, Java, Borneo) are relatively young, particularly Java and Sumatra, with west Java not emerging until the Mid to Late Miocene (Hall, 2013) and Sumatra going through uplifting events, owing to plate tectonics, as well as volcanism creating a series of islands with varying degrees of connectivity through the Oligocene and Miocene (Barber et al., 2005; Hall, 2013). More specifically, the early Oligocene saw Sumatra, and much of Java, connected to the Sunda shelf until in the early Miocene (Barber et al., 2005) they started disassociating from the mainland and from each other and submerging. Java became completely submerged through the Earliest Miocene, and Sumatra became a small collection of volcanic islands (Hall, 2013).

Geologic History

The Sunda region of Indonesia is also known as the ring of fire because of its vast volcanism. The volcanism is due to the extensive subduction zones at the edge of the Sunda shelf (Hall et al., 2009). One of the most famous volcanos of the region is Krakatoa, which most recently erupted in 2020, but has been known to have larger eruptions, such as that in 1883, measured at a six on the Volcanic Explosivity Index (Hall et al., 2009). Another volcano of the region known to have had a global impact is Toba. Toba had a mega eruption 17,000 years ago, and the abrupt

climate change it caused has been cited as a possible cause for the human genetic bottleneck, with ash deposits found even in Africa (Lane et al., 2013). The mega eruption has been noted locally on Sumatra to have affected the North-South distribution of some species (O'Connell et al., 2020; Wilting et al., 2012), while others were generally unaffected by the event (Louys, 2012).

Volcanic and uplifting events have been pointed as major drivers of speciation, particularly in the "more stable" tropics (Smith et al., 2014), and as uplifting events occur, multiple vicariance and dispersal events can occur over time (Hazzi et al., 2018). Given the age of the islands of the Sunda Shelf, the periods of connectivity both between and within islands, paired with uplifting events, multiple events have occurred that would drive speciation. Given the similar niches that would arise from such events and the time period covered, many of the mechanisms for cryptic speciation are in place. Cryptic species arise by three main means: 1) they are young species, 2) niche evolution, and 3) morphological convergence (Fišer et al., 2018). Cryptic species often get lumped into species complexes or a group of species sharing similar morphology, where species boundaries often remain unclear. The idea of species complexes predates that of molecular work used to delimit taxonomic groups by many years (e.g. (Inger, 1966).

The Sunda shelf has been a region of constant geologic change. In the Late Paleocene to the Early Oligocene, much of what makes up the current Sunda islands were above sea level and attached to the mainland (Barber et al., 2005; Hall, 2013). As the Late Eocene entered into the Early Oligocene subduction increased and the Makassar strait isolated West Sulawesi from the rest of the Sunda Shelf (Hall, 2013), effectively separating the Asian and Australia-Pacific biogeographic zones at Wallace's Line. By the Mid Oligocene (~30 mya), the lands of the Sunda

shelf had lowered significantly, with much of Java becoming a shallow sea (Hall, 2013). The Earliest Miocene still had North Sumatra joining the mainland but would separate into several smaller volcanic islands by the Early Miocene (~20 mya) (Barber et al., 2005; Hall, 2013). Java mainly had disappeared by this time. The Middle Miocene was marked by relatively high sea levels that would submerge Java and much of Northern Sumatra, with a volcanic island arc making up a significant part of the remaining fragments of Sumatra (Barber et al., 2005; Hall, 2013; Meijaard, 2004). The Sunda microplate rotated in the Late Miocene (~10 mya), causing further and more rapid subduction of the Indian plate, increasing both volcanic events and uplifting events along the what are now the Barisan Mountains in Sumatra (Barber et al., 2005). Java would also see these increased volcanic and uplifting events in the Late Miocene, with a significant part of West Java emerging in this time period as well with varying degrees of connectivity to South Sumatra and the mainland Sunda region (Hall, 2013; Meijaard, 2004). The Early Pliocene (~5 mya) would see the uplifts start to assemble the island arc of Sumatra into one landmass, with highlands along the west continuing to rise (Barber et al., 2005). The uplifting through the Early Pliocene would see Java form an island arc with western, central and eastern main islands (Meijaard, 2004), with central Java occasionally being completely submerged by high sea levels (Hall, 2013).

Highlands would continue to develop throughout the Pliocene, in Sumatra and with varying degrees of land emergent in West and East Java (Meijaard, 2004). The Early-Middle Pleistocene (~2-1.5 mya) saw varying degrees of connectivity between North Sumatra and the rest of the island, with the area around what is now the Toba caldera appearing to be where the disconnect of North and South occurred (Meijaard, 2004). Java was in most of its present day appearance, fully connected and with the outer Lesser Sundas all emerged as well. During the

Middle Pleistocene a global cooling even saw sea levels dramatically fall and connecting all of the Sunda islands to the mainland (Meijaard, 2004). Other global cooling events have continued to connect the Sunda Islands to the mainland occasionally since but, the uplifting events would continue to make isolated mountain habitat.

Species Ecology

Limnonectes kuhlii has been identified as encompassing cryptic species even before the common onset of genetic techniques (Inger, 1966). The utilization of genetic techniques was eventually used to identify 22 lineages, most of those previously unknown and within the *L. kuhlii* complex, and even recognizing several Javan and Sumatran clades using extremely limited sampling (McLeod, 2010b, 2010a; McLeod et al., 2011). Many of the species within the complex are found from mid to high elevation, suggesting low dispersal rates to disconnected mountainous regions. *Limnonectes kuhlii* are also heavily associated with aquatic environments, typically small rivers, but adjacent lentic environments have been observed to have them as well (Inger, 1966). Other anurans have been suggested to have high in-situ diversification, within islands, compared to dispersal between islands (O'Connell et al., 2018b). Ecological niches are likely conserved throughout Sundaic history, and therefore not likely strong drivers of speciation.

This study aims to establish relationships within the *Limnonectes kuhlii* complex using mitochondrial sequences compared to ddRADseq data, testing major clades for species within *L. kuhlii* and *L. sisikdagu*. From suggested species, divergence dating for all clades will be correlated to geologic events. Therefore, by utilizing gene-tree and species tree analyses paired with divergence dating, we answer the following questions: (1) How many species are present within the current confines of *L. kuhlii* on Java and *L. sisikdagu* on Sumatra? (1) When are

significant divergences occurring between and within Javan and Sumatran populations/species? (2) Do any major geologic or biogeographic events correlate with major splits within the *L. kuhlii* complex? We use the general lineage concept (GLC) (de Queiroz, 1999, 1998) to test and define species in this chapter. The integrative and pluralistic approach to use numerous criteria to define species allows for the identification of cryptic species, recognizing that the primary characteristic of a species existence is to be an independently evolving lineage (de Queiroz, 2007). By utilizing an integrative approach, our species delimitation paired with divergence dating correlated to biogeographic events allows for the discovery of species (Fišer et al., 2018)

Materials and Methods

Taxon Sampling and DNA Extraction

Our taxon sampling consisted of 81 individuals across Indonesia (Figure 3.1), including 30 samples from previous studies (Kurniawan, unpublished; McLeod, 2010b; McLeod et al., 2011). We collected and acquired tissue from them between the years 2012 and 2016. We extracted DNA from muscle or liver tissue stored in 1.5 mL of cell lysis buffer solution (0.5 M Tris/0.25% EDTA/2.5% SDS, pH = 8.2) using a phenol-chloroform protocol (Green and Sambrook, 2017; Sambrook and Russell, 2006). We checked the quality of our DNA extractions using a 1% agarose gel and quantified the DNA concentrations using an AccuGreen ™ Broad Range DNA kit (Biotium, Inc, Fremont, CA) on a QUBIT 2.0 Fluorometer (Life Technologies, Carlsbad, CA).
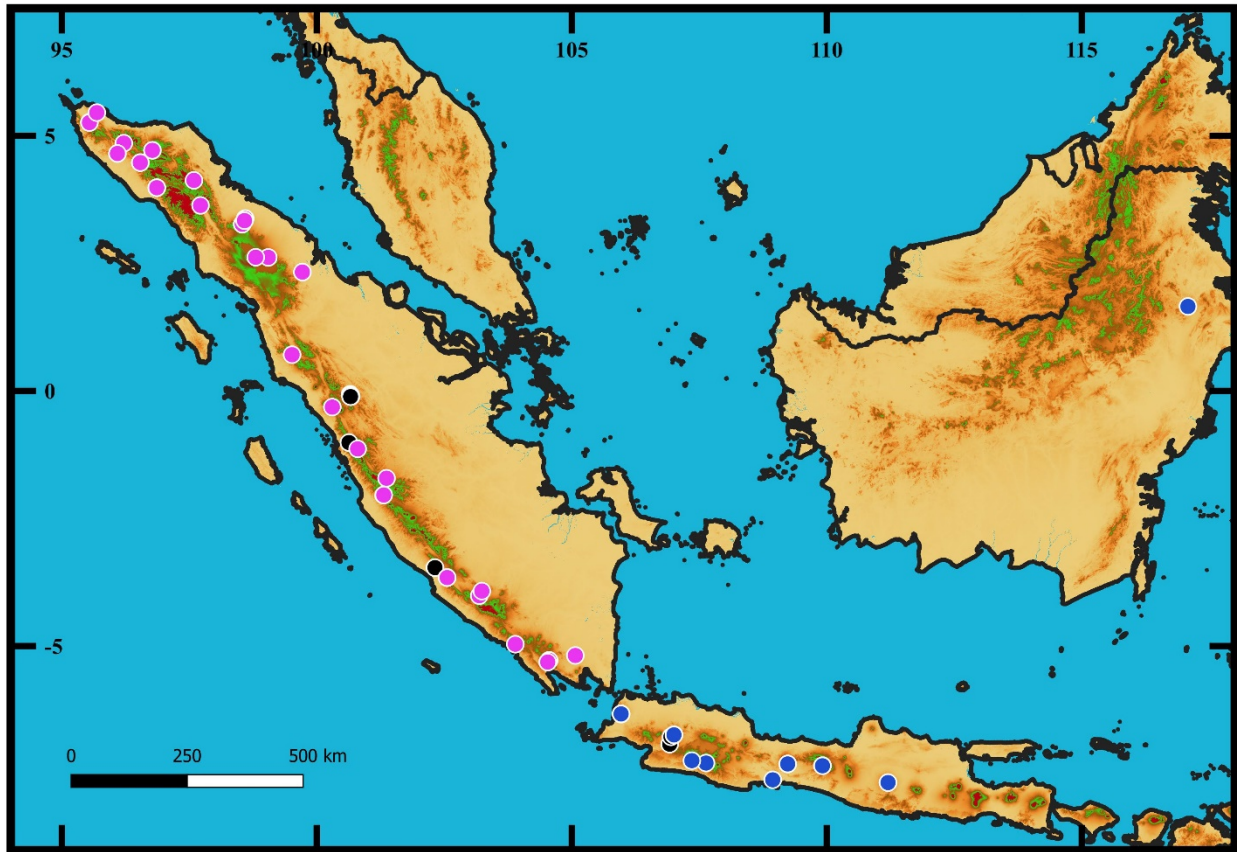
Figure 3.1. Sunda region of Indonesia with samples selected for Sanger sequencing. Black dots represent samples from McLeod (2010a, 2011), purple dots are from recent expeditions and are classified as *Limnonectes sisikdagu* and blue dots represent species from recent expeditions classified as *L. kuhlii*.

Once the DNA was extracted and quantified, we generated and processed the molecular data. We amplified a 616 basepair (bp) segment of the 16S large ribosomal RNA subunit gene and 512 bp of the 12S ribosomal RNA subunit gene for 51 individuals across Java and Sumatra (Figure 2.1) using standard primer pairs from Goebel et al. (1999), Palumbi et al. (1991), Palumbi (1996) and Wilkinson et al. (1996)(Table 2.1) in 25µL PCR reactions with an initial denaturation at 95°C for 2 min, followed by denaturation at 95°C for 30 s, annealing at 50°C for 35 s, and extension at 72°C for 2 min. We cleaned the PCR products using Serapure beads, following the Agencourt protocol (Beckman Coulter Co., Fort Collins, CO, USA) by Rohland

and Reich (2012). We then sequenced them in both directions using amplification primers and BigDye v3.1 (Applied Biosystems) on an ABI 3730 capillary sequencer (the University of Texas at Arlington Genomics Core Facility). We assembled the contigs and edited the resulting chromatograms in GENEIOUS PRIME v.2021.1.1 (Biomatters Ltd.). We added samples listed from McLeod (2010a)and McLeod et al. (2011) that we within *L. kuhlii* from Java and Sumatra to our 51 samples for a total of 81 samples and generated the multiple alignments of 1119 bp using the MUSCLE algorithm (Edgar, 2004) with default parameters. The outgroups included several samples from GenBank, including *Occidozyga laevis*, *Fejervarya vittigera*, *F. limnocharis*, *Hoplobatrachus rugulosus*, *Paa robertingeri*, *P. boulengeri*, *Limnonectes acanthi*, *L. arathooni*, *L. blythii*, *L. finchi*, *L. grunniens*, *L. gyldenstolpei*, *L. modestus*, *L. ibanorum*, *L. ingeri*, *L. kardasani*, *L. laticeps*, *L. leporinus*, *L. levtensis*, *L. macrocephalus*, *L. macrodon*, *L. magnus*, *L. microdiscus*, *L. microtympanum*, *L. palavensis*, *L. paramacrodon*, *L. parvus* (Appendix 1). Additionally, all newly sequenced samples have been deposited to GenBank.

Data for ddRadseq previously was generated for population-based analyses, see chapter 2. Dataset consists of 67 samples across Java and Sumatra.

Table 3.1. List of primers used in this study.

Gene Fragment	Primer name	Primer sequence	Primer source
12S	12S A-L	5'—AAACTGGGATTAGATACCCCACTAT—3'	Palumbi et al. 1991
12S	tRNA Val-H	5'—GGTGTAAGCGARAGGCTTTKGTTAAG—3'	Goebel et al. 1999
16S	16S L2510	5'—CGCCTGTTTATCAAAAACAT—3'	Palumbi 1996
16S	16SWilk2	5'—GACCTGGATTACTCCGGTCTGA—3'	Wilkinson et al. 1996

Species Analysis

We constructed phylogenetic trees for concatenated datasets of 12S and 16S fragments using maximum likelihood (ML) and Bayesian inference (BI) criteria. We used the program RAxML v8.2.12 (Stamatakis, 2014) with the GTR G + I model for ML analyses with a random starting tree and estimated all parameters using the CIPRES Science Gateway (Miller et al., 2010). We inferred the clade support values by ML analyses and estimated with the rapid bootstrap algorithm with 1000 replicates. We used BEAST v. 2.6.3 (Bouckaert et al., 2019; Suchard and Rambaut, 2009) to conduct BI analyses on the CIPRES Science Gateway (Miller et al., 2010). We utilized a single partition for these data. We conducted Bayesian analyses with random starting trees, ran for 20,000,000 generations with the Markov chains sampled every 1000 generations. We reviewed trace plots to ensure the convergence of Markov chain Monte Carlo (MCMC) runs and discarded a conservative 25% of the trees as "burn-in" once convergence was reached. We then created a consensus tree utilizing the software TreeAnnotator v.2.6.0 (Drummond and Rambaut, 2007) and visualized it in FigTree v. 4.4 (http://tree.bio.ed.ac.uk/).

Species Analysis

We generated species-level phylogenetic estimates utilizing three methods, Poisson Tree Process, Generalized Mixed Yule Coalescent, and Automatic Barcode Gap Discovery. Poisson Tree Process (PTP) is used to infer species boundaries by modeling speciation based on substitutions (Zhang et al., 2013); this model is considered comparable and can outperform the Generalized Mixed Yule Coalescent (GMYC). The GMYC has been shown to be a robust tool when looking at single-locus data (Fujisawa and Barraclough, 2013) through a likelihood methodology to delimit species. The third method utilized is Automatic Barcode Gap Discovery (ABGD), which functions as gap detection in pairwise differences distributions (Puillandre et al.,

2012). We utilized the bPTP server for PTP using default settings (Zhang et al., 2013). We utilized R version 3.0.2. (R Core Team 2013), with the packages paran (Dinno, 2018) and splits (Ezard et al., 2009) to run GMYC. We ran the ABGD on ABGD web (Puillandre et al., 2012), imputing a FASTA alignment under default settings.

Phylogenetic Relationships and Divergence Dating

We estimated the phylogenetic relationships and associated divergence times of individuals and suggested species groups for our mtDNA. We conducted Bayesian divergence dating analyses with our mtDNA data set using BEAST2 v2.6.3 (Bouckaert et al., 2019; Suchard and Rambaut, 2009). We performed analyses using an HKY model of nucleotide substitution, a constant size growth coalescent tree prior, and a log-normal relaxed molecular clock calibrated with a 1.3% per Myr rate of divergence (0.0065 clock rate; Macey et al., 2001). We ran this analysis with 20,000,000 generations with sampling every 1000 generations, producing a total of 10,000 trees. We assessed these runs using TRACER v1.6 (Rambaut & Drummond, 2009) to examine convergence. We discarded a burn-in of 10% and created a maximum clade credibility (MCC) tree with median heights from the remaining 9000 trees.

Results

Phylogenetic-tree Analyses for mtDNA

Topologies for ML and BI analyses were identical for both methods of analysis (Figure 2.3), with the ML score for RAxML at –8226.117024 for the 12S and 16S concatenated analyses. Results included several well-supported clades, three large clades: one for Java and two for

Sumatra, rendered clades for Sumatra polyphyletic. The more basal large Sumatran clade

consists of four strongly supported clades. One clade consisting of samples ENS 18077 and ENS

18532 originating from Bengkulu, Sumatra, one of the southernmost provinces. The other three

are from Aceh, the northernmost province of Sumatra. The larger Sumatran clade consists of

nine well-supported clades from all over Sumatra. This clade includes paratype specimens for

Limnonectes sisikdagu. The larger clades both stem from the Aceh Province of Sumatra. The

Java clade has one large supported split with several smaller well-supported clades. The large

split is between Eastern and Western Java, with smaller strongly supported divisions.

Species Delimitation

Species delimitation analyses for all three methodologies were much higher than population-

based analyses. GMYC yielded 24 species within the *Limnonectes kuhlii* complex within the

islands of Java and Sumatra (Figure 3.3, 3.4). PTP listed one fewer species, combining the clade

containing the paratypes of *L. sisikdagu* with its sister clade. The ABGD analyses recorded 24

species in a very different grouping than the previous two methods. The most significant

difference is seen in the Javan clade, where several species clade together in both GMYC and

PTP models but are single species. The clade with the paratypes of *L. sisikdagu* in the ABGD

model clades together with species clades 19-24 from the GMYC models. The GMYC and PTP

model species did seem to adhere to branches that were well supported. Some samples do appear

to clade with Javan clades, though they are in the southern province of Lampung on Sumatra

(ENS 14530) (Figure 3.4). For the most part, however, clades on Sumatra and Java clade

separately.

40
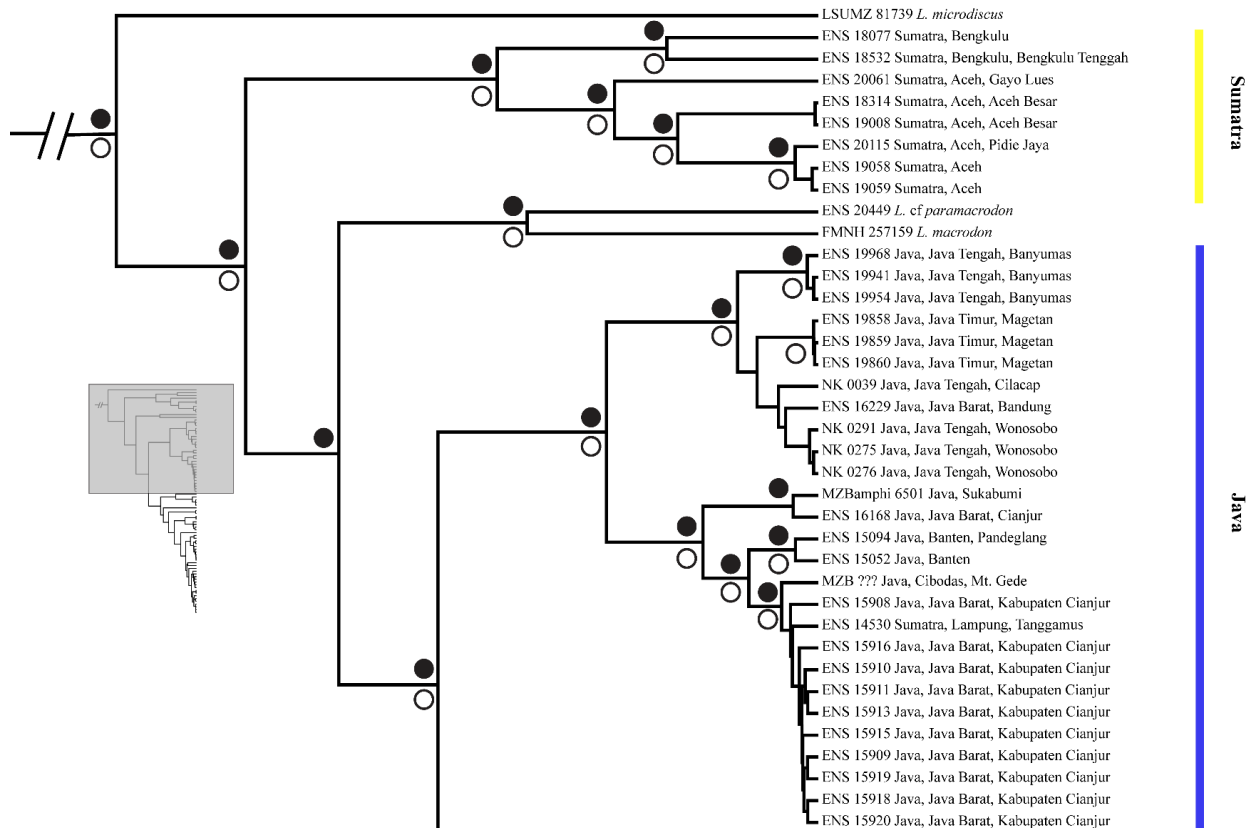
Figure 3.2. Bayesian tree for concatenated mitochondrial sequences 12S and 16S. Filled black dots represent high support for Bayesian Influence (> 0.95 posterior probability) and unfilled dots represent medium to high support for maximum likelihood (>80). Black bar denotes paratypes for *Limnonectes sisikdagu*.

41
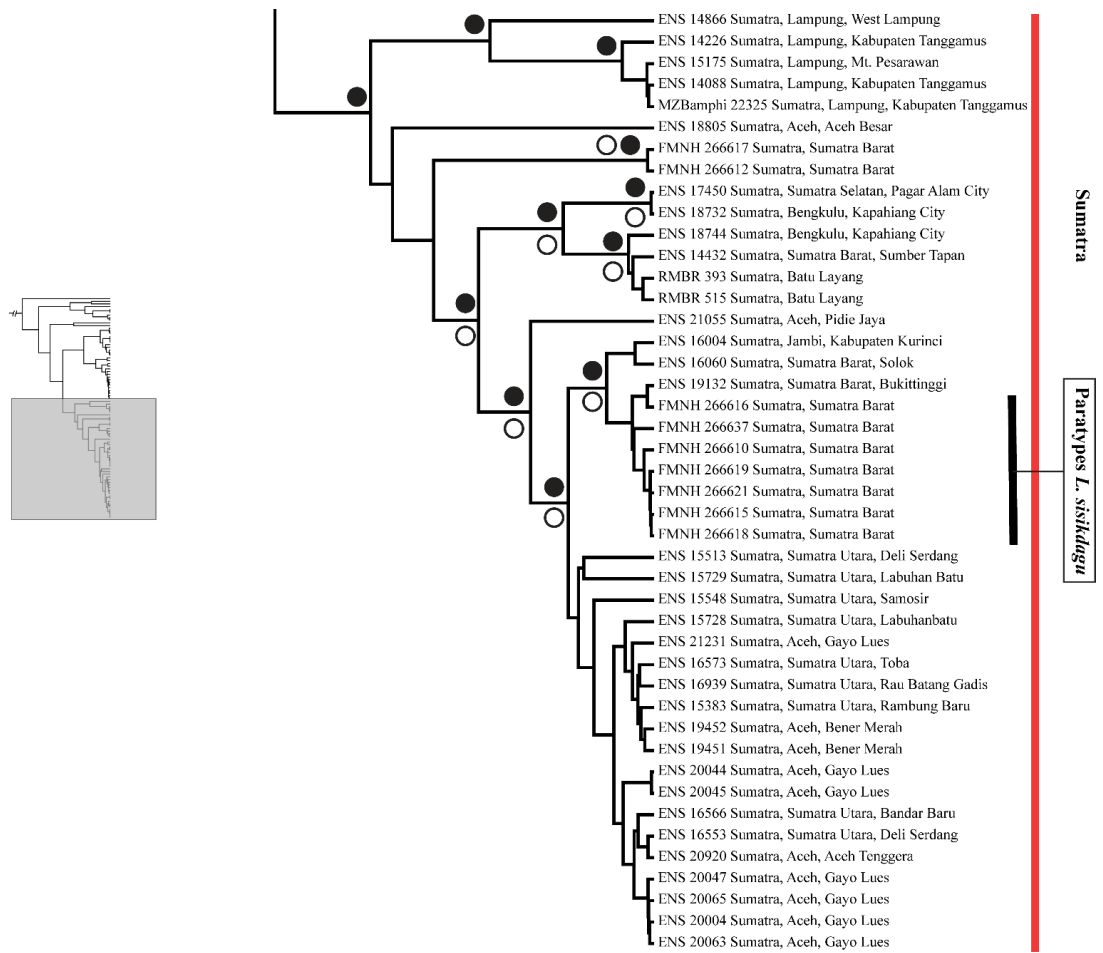
Figure 3.2. Cont.
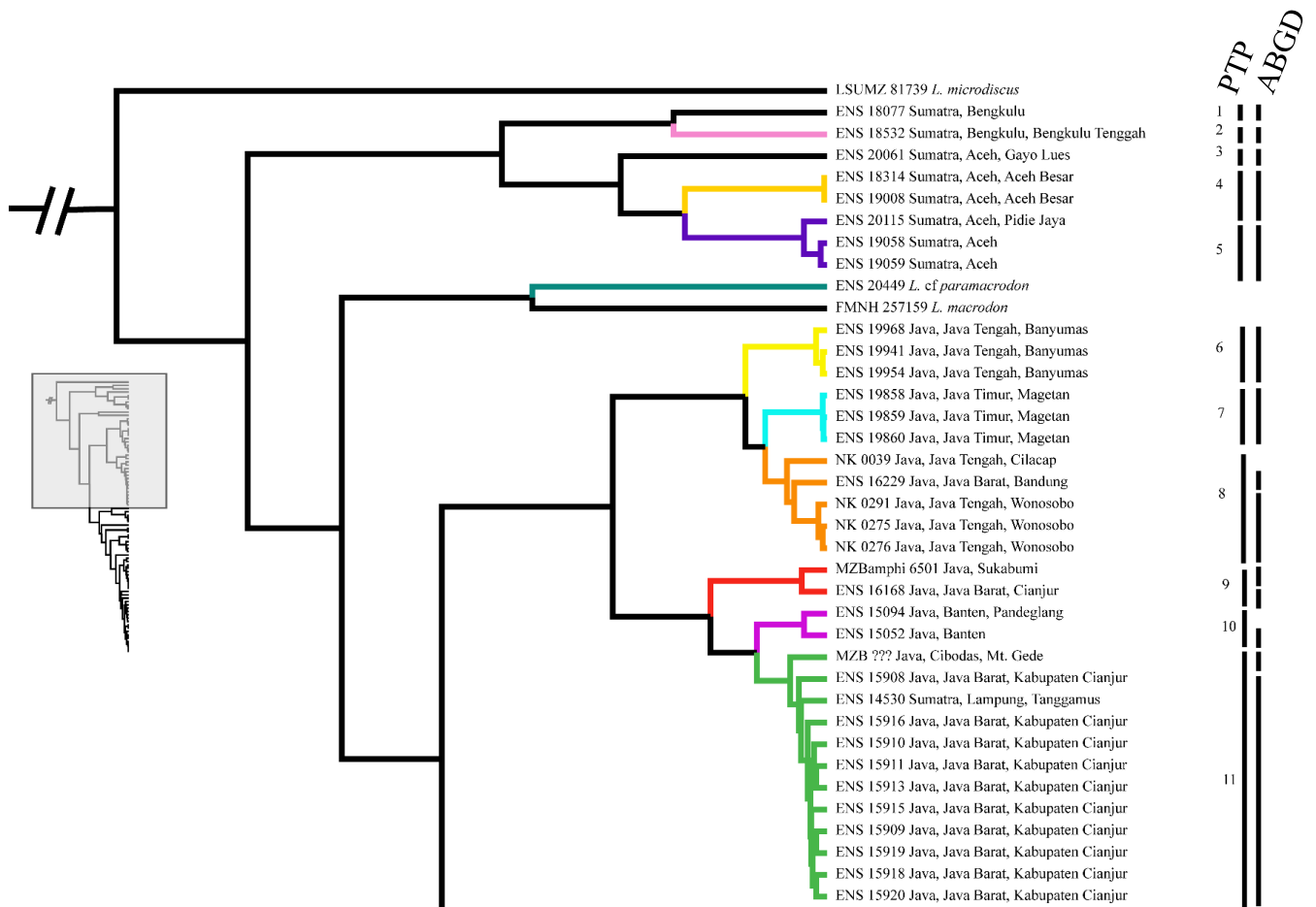
Figure 3.3. Bayesian tree based on mitochondrial sequences of 12S and 16S. Colors are associated with GMYC models of species delimitation. Bars are labeled for the methods used (PTP, ABGD). The number correspond to the number of species delineated with the GMYC model and correspond to Figure 3.4. Gaps in bar for ABGD are species not used for ABGD analysis.

Figure 3.3. Cont.
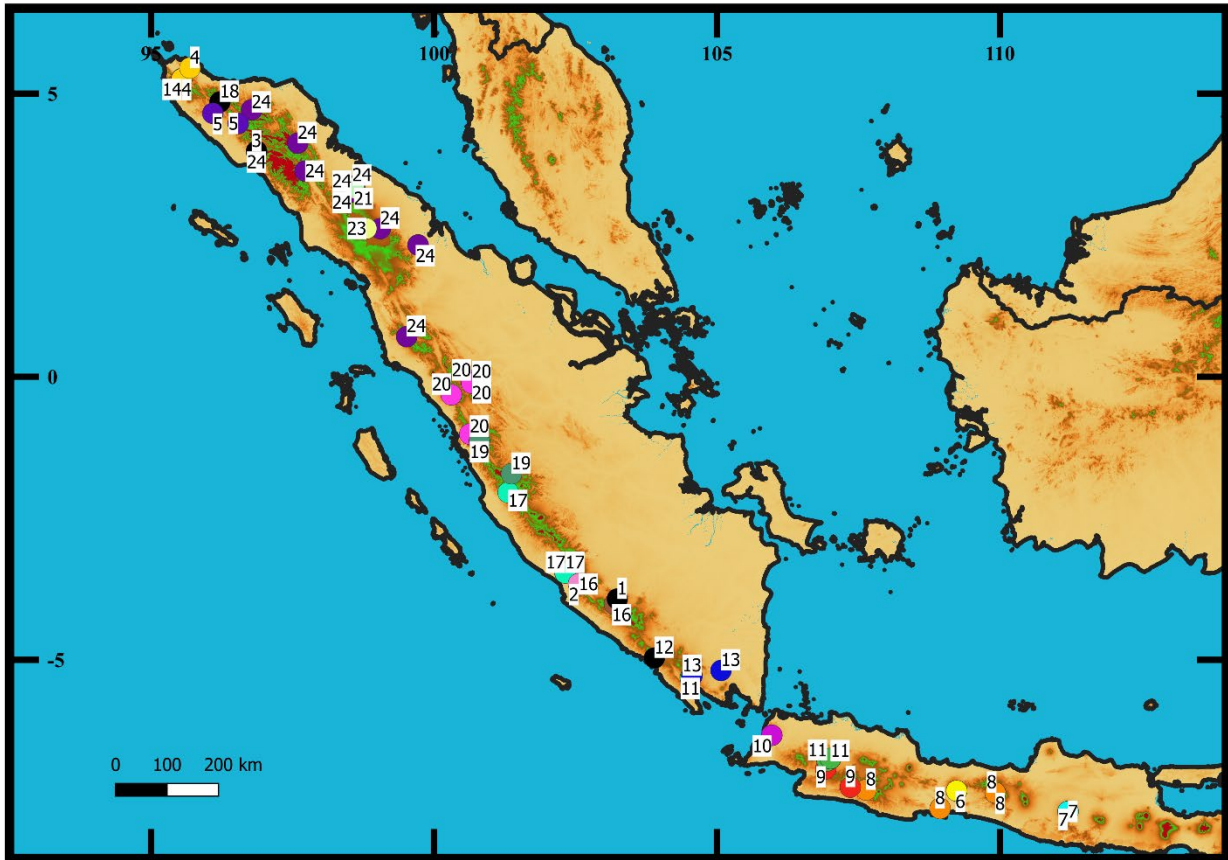
Figure 3.4. Map of species localities. Numbers and colors are associated to GMYC species clades to the tree in Figure 3.3.

Divergence Dating

Significant divergences for clades of *Limnonectes kuhlii* occurred in the Miocene (Table 3.2, Figure 3.3). The split between the *L. kuhlii* complex occurred approximately 17.4 mya (12.7-24.8 mya, 95% highest posterior densities [HPD]) in the Late to Mid Miocene. The division between Java and Sumatra occurred in the Mid Miocene at approximately 9.8 mya (7.3 -12.9 mya, HPD). The Java East-West split happened in the Late Miocene, about 5.4 mya (3.5-7.9 mya, HPD). The major spit within the Sumatran clade occurred in the Late Miocene, approximately 7.3 mya (5.3-9.8 mya, HPD). Further diversification occurred in the Pliocene and a few others in the early Pleistocene.
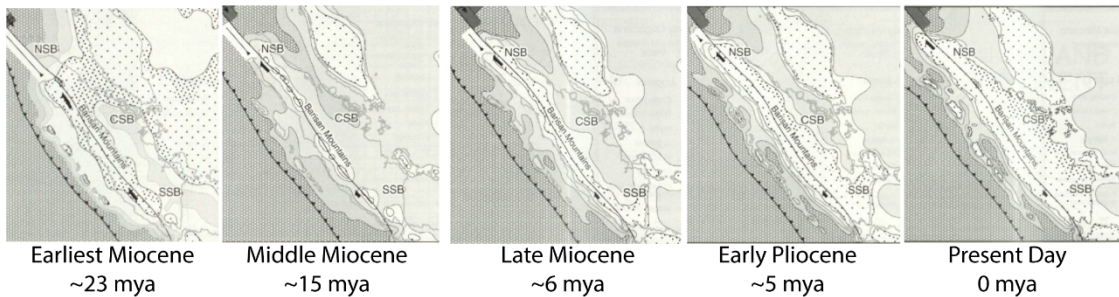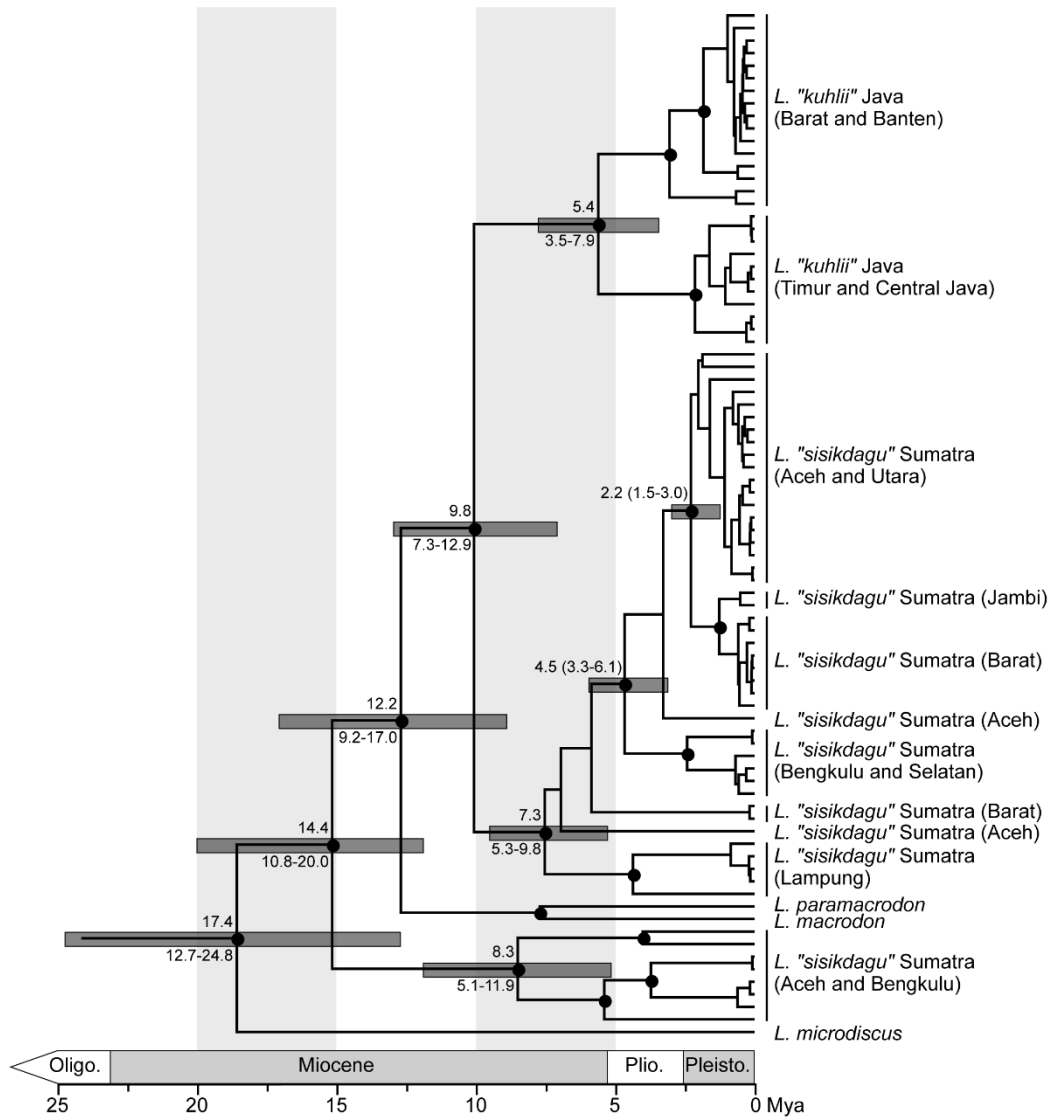
Figure 3.5. Divergence dating tree built in BEAST2. Nodes with dots are highly supported (> 0.9 posterior probability) and are correlated with Table 3.2 and Figure 3.3. Maps of Sumatra through time from Barber et al. (2005). Map symbols represented by close together circles and specks are costal land, dots evenly spaced are eroding areas, clear space neritic, slight shading upper bathyal zone, slightly darker shading middle to lower bathyal zone, grey with white dots deep sea, line with arrows represents spreading center and thin dark line fault zones.

Table 3.2. Molecular-clock time estimates for important nodes in the BEAST phylogeny of *Limnonectes kuhlii* complex on Java and Sumatra. Numbered nodes correspond to those shown in Figure 3.5. Data are median highest posterior densities (in million years ago), with ranges in parentheses.

Node	divergence date
Split between *L. microdiscus* and ingroups	17.4 (12.7-24.8)
Out *L. sisikdagu* and rest of ingroup	14.4 (10.8-20.0)
Major split within out Sumatran clade (clades 1,2 and 3,4,5)	8.3 (5.1-11.9)
Split between clades 1 and 2	3.9 (1.6-6.5)
Split between clades 3 and 4,5	5.3 (2.9-8.0)
Split between clades 4 and 5	3.6 (1.7-5.9)
between *L. macrodon* and large Java/Sumatra clade	12.2 (9.2-17.2)
Major split between Java and Sumatra	9.8 (7.3-12.9)
Major split between East and West Java	5.4 (3.6-8.0)
Split in East Java clades 6 and 7,8	2 (1.2-3.3)
Split in East Java clades 7 and 8	1.4 (0.7-2.3)
Split in West Java clades 9 and 10,11	2.9 (1.7-4.5)
Split in West Java clades 10 and 11	1.8 (1.0-2.7)
Split in Sumatra clades 12,13 and 14-24	7.4 (5.3-9.8)
Split between clades 12 and 13	4.2 (2.0-7.0)
Split between clades 14 and 15-24	6.3 (4.7-8.3)
Split between clades 15 and 16-24	5.4 (3.9-7.2)
Split between 16,17 and 18-24	4.6 (3.3-6.2)
Split between 16 and 17	2.4 (1.3-3.6)
Split between 18 and 19-24	3.2 (2.2-4.4)
Split between 19,20(*L. sisikdagu* holotypes) and 21-24	2.2 (1.5-3.1)
Split between 19 and 20(*L. sisikdagu* holotypes)	1.2 (0.7-2.0)
Split between 21,22 and 23,24	1.9 (1.2-2.6)
Split between 21 and 22	1.8 (1.2-2.6)
Split between 23 and 24	1.4 (0.9-2.0)

Discussion

Phylogenetic-tree Analysis

The phylogenetic tree generated from the mitochondrial fragments of 12S and 16S both

demonstrated the same three main clades (Figure 3.2) seen in the previous chapter from

ddRADseq data (Chapter 2). The topologies were not the same. The mitochondrial data put the

yellow clade (Figure 3.2) more basal in the tree than the samples for *Limnonectes macrodon* and

L. paramacrodon. Bayesian posterior probabilities and maximum-likelihood strongly supported

the same splits for the other clades. The overall trend still remained that, for the most part, clades

were either associated with Sumatra or Java with support for geographic regions. Data still

support an east-west division for Javan clades.

There are a couple of possibilities for the differences in topology between the ddRADseq

and mitochondrial datasets. One is mitonuclear discordance, where different patterns are shown

between mitochondrial and nuclear datasets causing disagreements in phylogeographic patterns,

populations, and species (Larmuseau et al., 2010); this can cause sex-biased biogeographical

differences between genomes (Toews and Brelsford, 2012). The pattern seen in the *Limnonectes*

kuhlii complex would not be the first case of mitonuclear discordance in *Limnonectes* but, causes

are still rarely explored (Abraham et al., 2021). Another possibility is that the two fragments of

12S and 16S used were not enough to resolve relationships fully. While both fragments are

common and well-used mitochondrial fragments, it is possible that they won't identify organisms

and their relationships correctly (DeSalle et al., 2005). The last main concern is the number of

samples (n). It has been demonstrated that not having enough individuals can result in an

inability to properly sort characters (DeSalle et al., 2005). One of the large differences between

the ddRADseq datasets and mitochondrial datasets are the number of species marked as

outgroups. While there were more outgroups for mtDNA, there were not many samples per species, usually, only one of any particular group, so their placement could be affected by the deficiency.

A sample from the southernmost province in Sumatra, Lampung (ENS 14530), was placed within the clade of Javan samples from Kabupaten Cianjur. The data for the Javan sample in the Sumatran clade from the ddRADseq data did not work for the mitochondrial sequencing but, it is interesting that the sequence from Lampung would clade in the same group as the Javan sample when it was restricted to just samples from Java.

Species Delimitation

Species boundaries were tested using three single-locus molecular species delimitation methods (GMYC, PTP, ABGD). All of which have commonly been used in conjunction with each other to evaluate single-locus datasets (Ellepola et al., 2021; Fouquet et al., 2021; Hofmann et al., 2019). Uncovering multiple species across Java and Sumatra was not surprising considering the numbers of identified species from other species groups that received better sampling for the region (O'Connell et al., 2018c, 2018a; Sarker, 2020; Shaney et al., 2020).

Both PTP and GMYC are considered robust methodologies for species delimitation, with PTP, occasionally considered to outperform GMYC slightly (Luo et al., 2018). The results of all species analyses suggest a minimum of 23 species (PTP) and a maximum of 24 (GMYC, ABGD) species. The species identified by GMYC will be discussed in conjunction with the PTP results, as the only difference is a combination of clade 19 and 20 into one clade. The outer group of Sumatra contained five identified species lineages, all with high support from BI and ML

methodologies on the gene tree (Figure 3.3). The Javan clade identifies three species for West Java and three species in East Java. The inner Sumatran clade contains types for *Limnonectes sisikdagu* and 11 or 12 previously unidentified species groups.

Given that all methodologies take in a gene tree as the assumption to accurately reflect species diversification, the number of species could be an artifact of the gene tree. However, given that GMYC is often expected to over split and ABGD typically under splits (Luo et al., 2018), clades may likely reflect species. The species are restricted to generally small geographic localities (Figure 3.4). Many of these groups correspond to the populations that were presented in the previous chapter with ddRADseq data.

The species should be further evaluated using both dating estimates and biogeographical comparisons. These would strengthen the evidence to recommend elevation to species designation. The current understanding of species in the *Limnonectes kuhlii* complex is that they are morphologically conserved between species. Discrete characters such as skin roughness patterns and a rough patch on the chin differentiated *L. sisikdagu* from *L. kuhlii* (McLeod et al., 2011), and initially, they were highlighted by phylogenetic differences (McLeod, 2010a). It may not be unfathomable that morphological characters thought to be variable within a species are actually traits that identify a species group. With consistent concerns for protecting biodiversity across Southeast Asia (Hughes, 2017), over splitting may be a solution to combat biodiversity loss.

Divergence Dating and Biogeography

Our results for the *Limnonectes kuhlii* complex are congruent with other studies focusing on reptiles and amphibians from Indonesia (O'Connell et al., 2018c, 2018a; Oliver et al., 2015), with several areas correlating to geologic and climatic events.

The Javan and Sumatran *Limnonectes kuhlii* complex initially split from the rest of *Limnonectes* sometime in the Late Oligocene (~25 mya) to Mid Miocene (~15 mya). During this time, Sumatra started to separate from peninsular Malaysia (Barber et al., 2005). The Earliest Miocene saw regional sag through the Mid Miocene. The sinking of land left the Sunda Shelf fragmented in several small islands, particularly Sumatra, fragmentation exacerbated in the Middle Miocene when sea levels rose (Meijaard, 2004). The Mid Miocene also began the re-activation of faults as the Indian tectonic plate began refolding base sediments under the Sunda microplate, possibly causing the rotation of Sumatra and Borneo (Barber et al., 2005), which is considered one of the sites of high diversification for *Limnonectes* (de Bruyn et al., 2014). Species clades 1, 2, 3, 4, and 5 would have separated from the lower clades at this time, likely due to island separation around 14.4 mya. As the island isolations continue, clades 6-24 would separate from *L. macrodon* and *L. paramacrodon* around 12.2 mya. In the Mid to Late Miocene (~10 mya), Sumatra is connected with the mainland in two places, one in the north and one in the south. More importantly, at this time, we see west Java develop a significant landmass as uplifting events continue, and we see a split between the Javan clade (species 6-11) and the Sumatran clade (species 12-24) (Figure 3.5, Table 3.2, clades associate with Figure 3.4 and 3.3).

Several populations would have been isolated on volcanic islands by the Mid Miocene. In Mid to Late Miocene, Western Java was above sea level due to uplifting events; East Java would follow by the end of the Miocene and connect with Western Java (Hall et al., 2009). (Figure 3.5,

Table 3.2). The global cooling events of the Late Miocene (Herbert et al., 2016) in tandem with uplifting events (Barber et al., 2005; Hall et al., 2009) would allow for more land to surface not only connecting Sumatra again, also connecting the rest of the Sunda islands to the Mainland. At this time, we see major divergences in both the Javan *L. kuhlii* clade and the Sumatran one. The uplifting paired with lowering sea levels would see a split at around 8 mya within the basal Sumatran clade, with species 1 and 2 in Bengkulu becoming isolated from species 3-5 in Aceh. In the large Sumatran clade, we observe species 12 and 13 separating from the rest of the species in the red clade. The barrier may have been in response to uplifting events or that Sumatra has varying degrees of separation from the mainland at this time. One thing is very clear some events separated northern species clades from southern species clades in the Late Miocene (~8.5 - ~7 mya) (Figure 3.5, Table 3.2). At the very end of the Miocene (~5.4 mya), we see a decisive split between eastern and western Java (Figure 3.5, Table 3.2). Species 6-8 would be species found in only Central and East Java, and species 9-11 would only be found in West Java (Figure 3.3, Figure 3.4., Figure 3.5).

East and West Java are geologically distinct from each other, with Central Java also having some geologic differences to set up some semblance of a boundary (Hall, 2007). On the other hand, Sumatra has a long fault system running North to South across the island. There is debate regarding whether or not continuous uplifting happened across the fault (Barber and Crow, 2005); the current diversity within the *Limnonectes kuhlii* complex on the island does suggest that the uplifting events were neither continuous nor consistent between areas of the uplift zone.

Global cooling continued through the Pliocene, reaching similar average temperatures to the modern era (Burke et al., 2018). The Early to Middle Pliocene (~5-3 mya) saw high seas in

the Sunda region, separating Sumatra into North, Central, and South portions, with the South

portion having a land bridge between the Malaysian peninsula and Borneo (Meijaard, 2004)

which have since separated. Java, meanwhile, was also separated into a larger western island

with multiple smaller central and eastern islands (Meijaard, 2004). Volcanism regularly occurred

across both Java and Sumatra as the Sunda microplate rotated, causing more active subduction

across the Indian plate and increasing volcanic activity, along with growing uplifting events in

North Sumatra specifically (Barber et al., 2005). Volcanic and tectonic uplifting would create

increasingly isolated environments. As the species that raised with the uplifting event became

acclimated to their increased elevation, their ability to traverse the distance between similar

habitats diminished, creating isolated populations as the events continued. With the uplifting

zones and high sea levels, multiple routes of dispersal were cut off, allowing the possibility of

multiple clades to become isolated populations. These continued uplifting events would

eventually isolate populations from each other. The few species in sympatry likely dispersed

after volcanic events allowed for recolonization, or if a major uplifting event connected nearby

mountain ranges as seen in species clades 4 and 14 as well as 21, 23, and 24.

Conclusions

The Sunda Shelf has had a remarkable history of geologic activity, creating a wide variety of

habitats that harbor numerous unidentified species. As a poorly explored region of the world

(Iskandar 2004), there is much that might yet be uncovered. Cryptic species such as those in the

Limnonectes kuhlii complex are cryptic because they are relatively young. Habitats have only

recently separated many of the species, only separating species just over 1 mya, giving time for

genetic divergence but not enough for morphological divergence as highland species that

continually adapt to rising elevations. The river habitats for which *Limnonectes* prefer likely did not change with uplifting events, so morphological convergence is an unlikely event to happen.

From the rotation of the Sunda microplate, the tectonic and volcanic activity levels continue through to today, with consistent volcanic activity and uplifting events still occurring regularly (Barber et al., 2005; Whelley et al., 2015). Isolated mountain ranges continued through the Pleistocene when more continuous high elevation regions started to form, allowing high elevation species to come back into contact with one another in some cases. At the last glacial maxima, the entire Sunda shelf was connected (Sumatra, Java, Borneo) (Meijaard, 2004). Lower elevation species would be able to widely disperse across the region, as shown by species widely distributed across islands, such as *Rhacophorus pardalis* (Frost, 2021). The *Limnonectes kuhlii* species complex would not disperse, as they are primarily restricted to higher elevation environments and river drainages.

Understanding how the biogeographic history of Indonesia relates to phylogenetic history and speciation in the *L. kuhlii* complex is important. Whether uncovered species continue to be of Least Concern or not, they are still declining through most of the range (van Dijk et al., 2004). This work will contribute to a taxonomic update, at which point a true assessment of the *Limnonectes kuhlii* complexes species richness can take place for Java and Sumatra. This is critical in the Anthropocene, where understanding the true diversity of a region is critical for conservation efforts.

Chapter 4: Intrasexual Selection Drives Morphological Differences in Males of Closely Related Species Within the *Limnonectes kuhlii* Complex (Anura: Dicroglossidae)

Introduction

Sexual dimorphism is well documented in anurans, with females generally being larger than males. This typical anuran dimorphism equates to larger females laying more eggs (Kupfer, 2007; Shine, 1979). However, in the *Limnonectes kuhlii* species complex, the males are larger than the females, due mainly to a larger head (Emerson and Inger, 1992). Males are also noted to have larger fangs , and a frontoparietal medial ridge developed to varying degrees exclusively by males (McLeod, 2010a). These morphological features are often a product of sexual selection, which operates through two classically defined mechanisms: male-male combat and female choice (Darwin, 1859). Female choice is intersexual selection where the female chooses the "best fit" male based on a set of morphological traits or behaviors (Holman and Kokko, 2014). The intrasexual selection of male-male combat can work in tandem with female choice (McCullough and Simmons, 2016), or with less female choice directly involved, such as competition for territory or breading habitat not in view of female species (Candolin and Voigt, 2001).

Intrasexual selection is often achieved through male-male combat for access to females or breeding habitat, though females typically will still have final say on whether or not a mate is appropriate (Jones and Ratterman, 2009). Much of the selection in anurans is equated to intersexual selection, with females choosing the partner based on various displays, such as vocalizations. In situations such as these communal breeding ponds, the size of the male is not

thought to directly matter to mating success, whereas it matters for the purposes of fecundity in females (Kupfer, 2007).

The genus *Limnonectes* is known to utilize several different mating strategies. In *Limnonectes palavanensis*, females have been documented advertising to males for partner selection (Vallejos et al., 2017) as opposed to the advertisement burden being lain upon the males. In another species, *L. larvaepartus*, internal fertilization takes place (Iskandar et al. 2014). Species within the fanged-frog cryptic species complex *L. kuhlii* demonstrate intrasexual selection through male-male combat for the best brooding territory (Tsuji and Matsui, 2002). A larger head typically leads to more combat wins and, subsequently, more reproductive opportunities for the victorious males (Tsuji and Matsui, 2002).

In my chapter 3 we explored the task of identifying species within the *Limnonectes kuhlii* complex on the islands of Java and Sumatra. An estimated 19 to 24 species were identified from what had been described previously as two to three species in Java and Sumatra (McLeod, 2010a), and even prior to genetic means being readily available, populations within the *L. kuhlii* complex were thought to contain multiple species (Inger, 1966). Species within the *L. khulii* complex are considered to be morphologically similar across lineages, leading to difficulty in identifying even closely related species within the greater *Limnonectes* genus. The difficulty in identifying species not only impairs our ability to understand the evolution of the Sunda Region of Southeast Asia, but can have conservation implications on species "hidden" within the complex that may be in small ranges or sensitive regions (Angulo and Icochea, 2010). As the islands of the Sunda shelf are relatively young (25–10 my), species would be young and not have morphologically differentiated from its progenitors.

Size discrepancy, and other secondary sex characteristics, such as larger odontoids as seen in the genus *Limnonectes*, often present themselves in males of species that partake in male-male combat (Shine, 1979). Intrasexual selection over generations would exacerbate traits on the males much more than the females, since such traits as a larger head and more developed frontoparietal medial ridge would win them breeding territory. Females, however, would not be subjected to such selectivity of traits. In the *L. kuhlii* complex, males are larger than females due to male-male combat. This puts more sexual pressure on males than females. Sexual selection rapidly driving morphological change has had an effect on many species, with males leading accelerated rates of evolution (e.g. octopus [Ibáñez et al., 2019], bovids [Reuland et al., 2021]) and experiencing stronger selection. Birds are likely a good model here.

This study aims to highlight how intrasexual selection would act in a noticeable way on males but not in females of closely related species. It is known that morphological dimorphism between males and females separates the sexes, but it is not know to what extent sex-specific differences separate species. If certain traits in males give them access to more breeding areas, these traits would become dominant within a population and lead to other measurable traits within males of a species, but not within females of the species. Thus, I have three aims in this chapter: first to characterize morphological differences between males and females of two closely related species, predicting greater dispersion between the males of these two species than the females. Second, to identify key morphology traits that are driving these differences, and to relate these traits to the ecology of the species. Finally, I will relate these morphologic differences to the biogeographic history of the *Limnonectes kuhlii* complex.

Materials and Methods

Our specimen sampling for this study consisted of 21 adult individuals across the islands of Java and Sumatra, Indonesia (Figure 4.1). For most of these tissue was collected (between 2012 and 2016) and phylogenies were reconstructed using nuclear (ddRADseq) and mitochondrial (12S
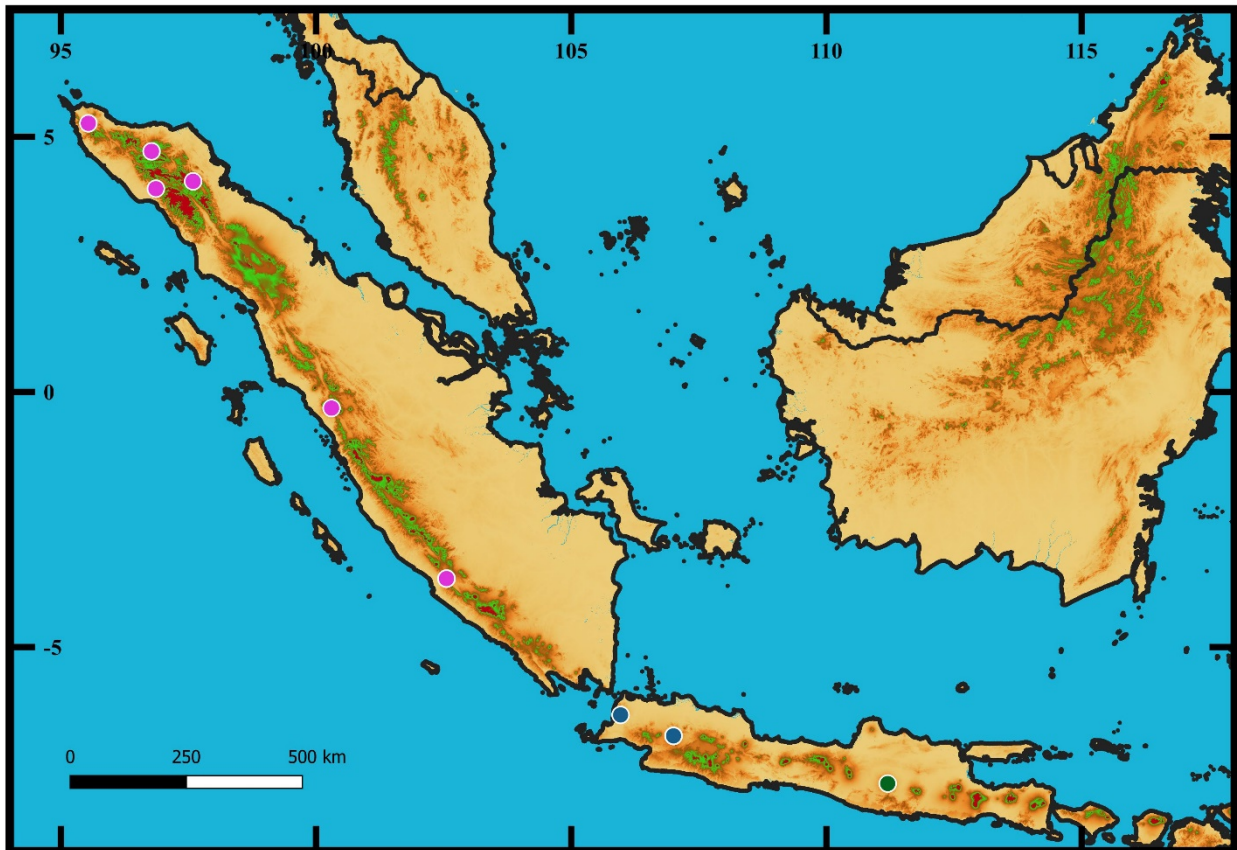
Figure 4.1. Localities of samples measured in ImageJ. Pink are Sumatra samples, blue and green are Java samples.

and 16S) markers to determine species and clade belonging (see chapters 2 and 3 of this dissertation for molecular protocol details). A few samples measured for morphological traits also originated from localities represented by sequences, and were included to increase the sample size. We used the following adult specimen: nine females from Java (ENS 15907–11, 15913, 15919–20), six females from Sumatra (ENS 18732–33, 18744, 19452, 20065, 21231), three males from West Java (ENS 15094, 15916–17), one male from East Java (ENS 19858), and

two males from Sumatra (ENS 18805, 19132). Measurements of similar values were checked running a regression analysis for outliers. Outliers were a female sample from Sumatra (ENS 18733) and the East Java male (ENS 19858) were removed from final dataset. Figures 2.2 to 2.4, and 3.2 to 3.3 show the placement of the samples used in this chapter for external and internal morphological analyses in terms of their population belonging, phylogenetic reconstruction, and species delimitation.

Samples were sexed based on their head size, fang length, and by creating an incision to check gonads and notice the presence of eggs in females. Data consisted of 12 measurements of the skull (Table 4.1), since that is highlighted as the primary factor in determining the victor in male-male combat (Tsuji and Matsui, 2002). The data were analyzed through Project R 3.6 (R Core Team, 2018) in RStudio Build 351 (RStudio Team, 2021) using a principle component analysis (PCA) in the package 'ggplot2' (Wickham, 2016) for visualization. Since all differences between species can be part of the biologically relevant phenotype (Setiadi et al., 2011), including overall size, measurements were not corrected for size (Bendik et al., 2013). Three PCAs compared males to females, males to males, and females to females. Three more PCAs were run for all datasets following a general linearized model to correct for body sizes' influence on measurements of the skull, following a more traditional approach.

We analyzed ten individuals using Computerized Tomography (CT) scan analysis (Figure 4.2) from two different Sumatran locations (West Sumatra and Aceh Province). The data set consisted of three females from Aceh (ENS 19357, 20016, 20105), two females from West Sumatra (ENS 14425, 19715), three males from Aceh (ENS 18805, 20043, 21215), and two males from West Sumatra (ENS 19132, 19166) (Figure 4.2). We CT scanned the samples using the Shimadzu inspeXio SMX-100 CT scanner. Subsequently, we reconstructed the raw X-ray

data using Shimadzu's inspeXio software. We rotated and cropped our resulting image stacks in

ImageJ and imported them into the open-source program Drishti v.2.0. From the 3D surfaces, we

placed 43 landmarks using Stratovan Checkpoint and exported them as NTS files (Figure 4.7).

The landmarks we used were a subset of those used in Tokita et al. (2018), along with others
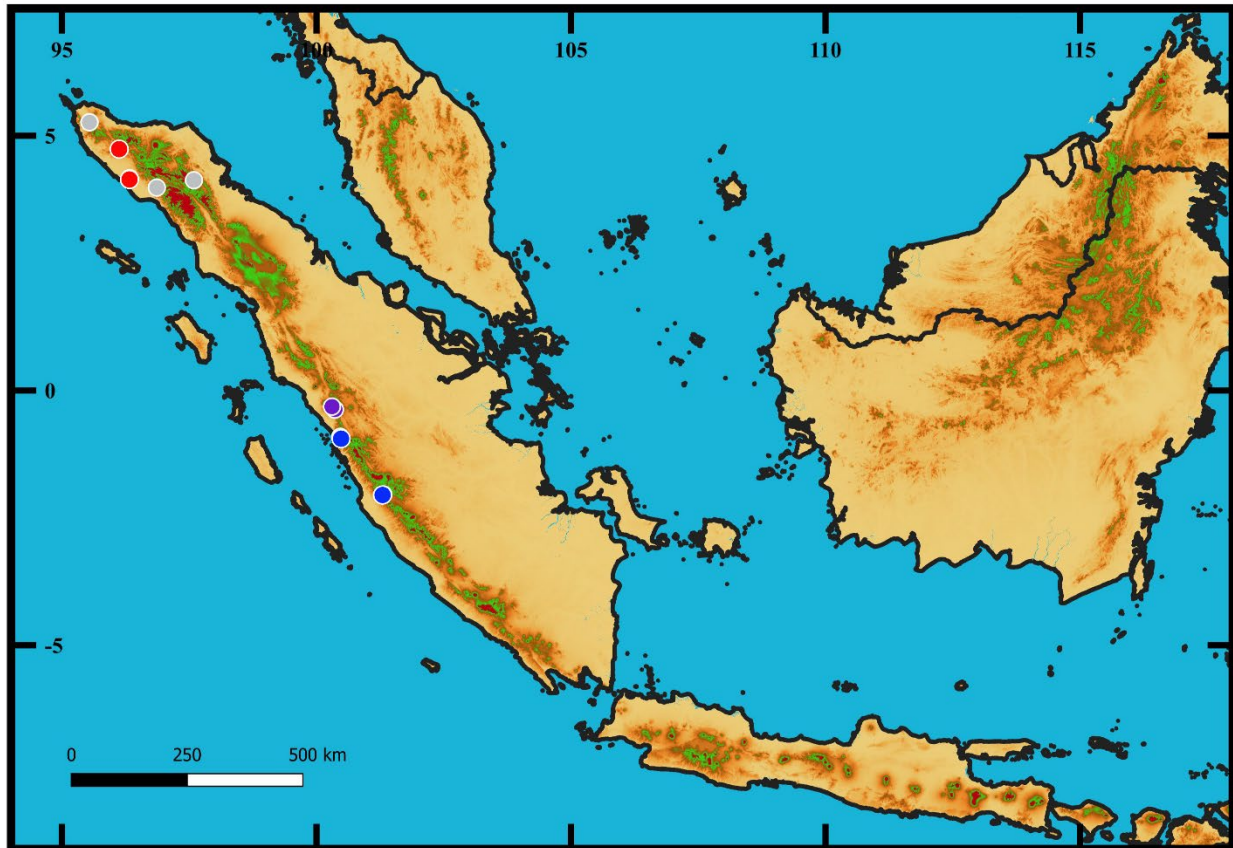
Figure 4.2. Localities for ct-scanned Morphometric analysis. Grey are males from Aceh, purple are males from West Sumatra, red are females from Aceh, and blue are females from West Sumatra.

placed for genus specific traits, such as the large odontoids and the frontoparietal medial ridge.

We subjected the resulting landmark data to a generalized Procrustes analysis in R in order to

obtain shape matrix accounting information related to position, orientation, and scale.

Additionally, we ran a PCA and associated wrap figures using the 'geomorph' R-package

(Adams et al., 2021; Baken et al., 2021).

Results

External Morphology Analysis

For the external measurements of 21 samples (Table 4.1) males, on average, were always larger than females in key skull measurements, most notably the overall size (SVL), head length (HL), head width (HW), and interocular distance (IOD). Many of the other traits of the skull were variable between the sexes of the respective species. The largest female was never larger than the largest male for species with both sexes represented.

The regressions performed on measurements, against each other for the purpose of data curation, revealed that one female sample from Sumatra (ENS 18733) and one East Java male (ENS 19858) were outliers. The two specimens were then removed from the data sets for PCA analyses. For the combined dataset comparing males to females corrected by overall body size (SVL), clear separation between the sexes is shown with PC1 explaining 79.62% of differences and PC2 explaining 8.5% for a total of 88.12% of the variance being explained by the PC1 and PC2 (Figure 4.3, Table 4.2) for those corrected by overall body size. The residuals for head width as a factor of overall body size explained the majority of the influence followed by the residuals for head length as a factor of overall body size. For values uncorrected by total body size (SVL), PC1 describes 69.58% of the variance and PC2 describes 9.68% of the variance (Figure 4.4, Table 4.3). The loadings for head width, head length, and head length on a diagonal influence the variance seen along PC1 the most, with other measurements having modest influence on the plot.

For the male-to-male comparison, we used two males from Sumatra and three from Java. The sample from East Java was removed as it was considered an outlier. All males demonstrated

clear separation from each other, with PC1 describing 87.42% and PC2 describing 5.73% of the analysis (Figure 4.5, Table 4.4). For values uncorrected for using total body size (SVL), PC1 describes 47.63 % of the variance and PC2 describes 38.94 % of the variance (Figure 4.6, Table 4.5). The loadings for head length on a diagonal influence the variance seen along PC1 the most and the loadings for snout to nostril distance, head length, and head width influencing the most variation across PC2. Slight differences across both PC1 and PC2 separate the males from each other in this PCA.

The female-to-female comparison had the largest sample size for single sex comparison, with six for the Sumatran clade and nine for the Javan clade. The females overlapped with samples from West Java being well within the main grouping of samples from Sumatra (Figure 4.7, Table 4.6). In the analysis PC1 explains 82.24% of the variability and PC2 5.93% for a total of 88.17% explained by the skull measurements as a factor of body size. For values uncorrected for using total body size (SVL), PC1 describes 44.93% of the variance and PC2 describes 26.83% of the variance for a total of 71.76% of the variance described by the PCA (Figure 4.8, Table 4.7). There is significant overlap of the two species within the plot with both PC1 and PC2 showing the strongest influence from head width, head length, and head length on a diagonal.

Table 4.1. Measurements of the *Limnonectes* skull. SVL (snout-vent length, HW (head width), HL (head length), HLd (head length diagonal), TYM (tympanum diameter), HWn (head width at nostril), IND (internarial distance), SEM (snout extend past mouth), ED (eye diameter), IOD (interocular distance), NAE (nostril to anterior eyelid), SN (snout to nostril distance), TEY (tympanum eye distance).

Species Group	N	SVL	HW	HL	HLd	TYM	HWn	IND	SEM	ED	IOD	NAE	SN	TEY
Sumatra Male	2	77.55 (74.29-80.81)	35.58 (34.67-34.49)	31.67 (30.79-32.56)	33.53 (31.89-35.17)	2.64 (2.16-3.12)	8.26 (8.15-8.38)	3.89 (3.38-4.40)	3.21 (2.99-3.43)	4.91 (4.10-5.72)	6.97 (6.85-7.08)	8.14 (5.62-10.66)	12.23 (11.68-12.79)	2.26 (1.90-2.62)
Sumatra Female	6	55.56 (42.11-61.53)	23.73 (16.71-32.45)	19.00 (16.35-21.03)	22.42 (17.63-26.34)	3.10 (2.67-3.48)	6.40 (4.13-7.71)	4.12 (2.86-5.36)	2.59 (1.48-3.77)	4.71 (3.71-6.57)	5.80 (3.49-8.27)	5.00 (3.13-9.03)	4.54 (2.42-11.82)	2.71 (2.37-3.45)
Java Male 1	3	71.45 (65.49-76.86)	32.60 (30.0-34.09)	28.05 (24.22-33.79)	29.69 (20.98-36.1)	3.12 (1.98-5.19)	8.03 (5.29-9.48)	5.50 (3.67-6.72)	2.64 (2.29-3.11)	4.89 (3.78-7.0)	7.51 (6.58-8.05)	6.39 (6.0-6.84)	2.5 (1.55-3.91)	3.92 (3.01-5.49)
Java Female	9	63.683 (48.89-75.64)	24.33 (19.89- 27.6)	17.14 (11.85-24.55)	22.04 (14.44-26.20)	2.91 (1.98-4.22)	8.66 (6.5-10.62)	4.84 (3.06-6.34)	2.28 (1.03-3.41)	4.75 (2.9-7.32)	6.17 (4.87-9.21)	6.00 (4.03-7.20)	3.34 (2.36-4.44)	4.10 (1.21-6.21)
Java Male 2	1	53.79	23.27	21.25	21.92	3.53	9.14	4.31	2.22	6.11	4.99	4.04	3.38	3.72
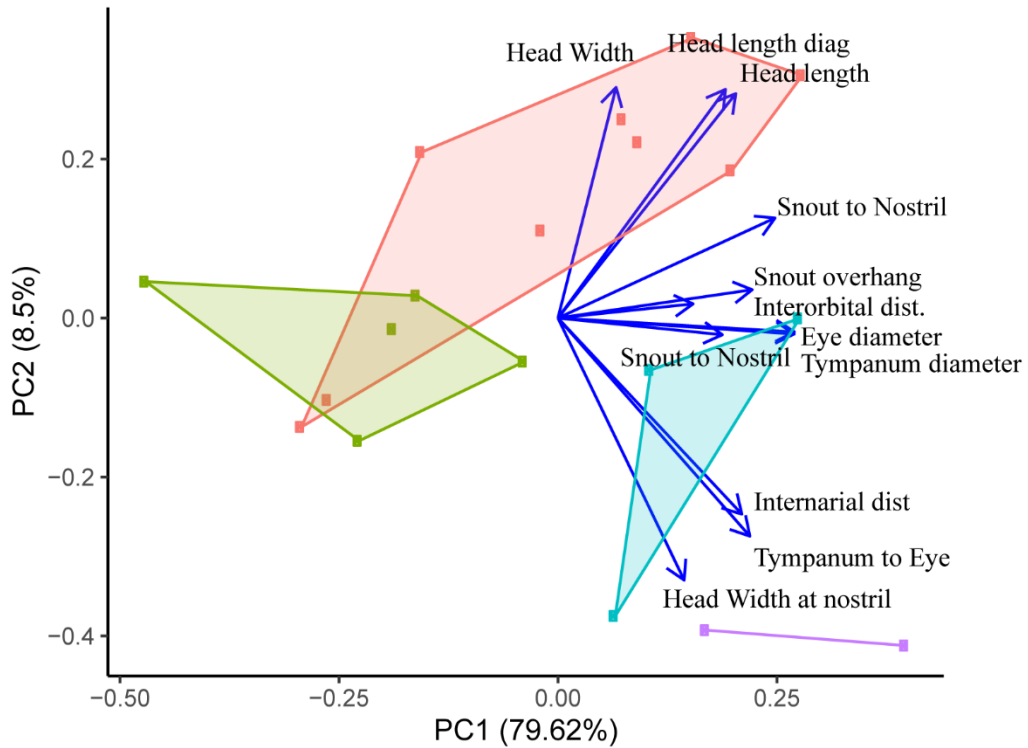
Figure 4.3. PCA plot of samples measured for this study corrected by total body length. Blue are males from Sumatra, purple are males from Java, green are females from Sumatra, and red are females from Java. Loadings and Eigenvalues are in Table 4.2.
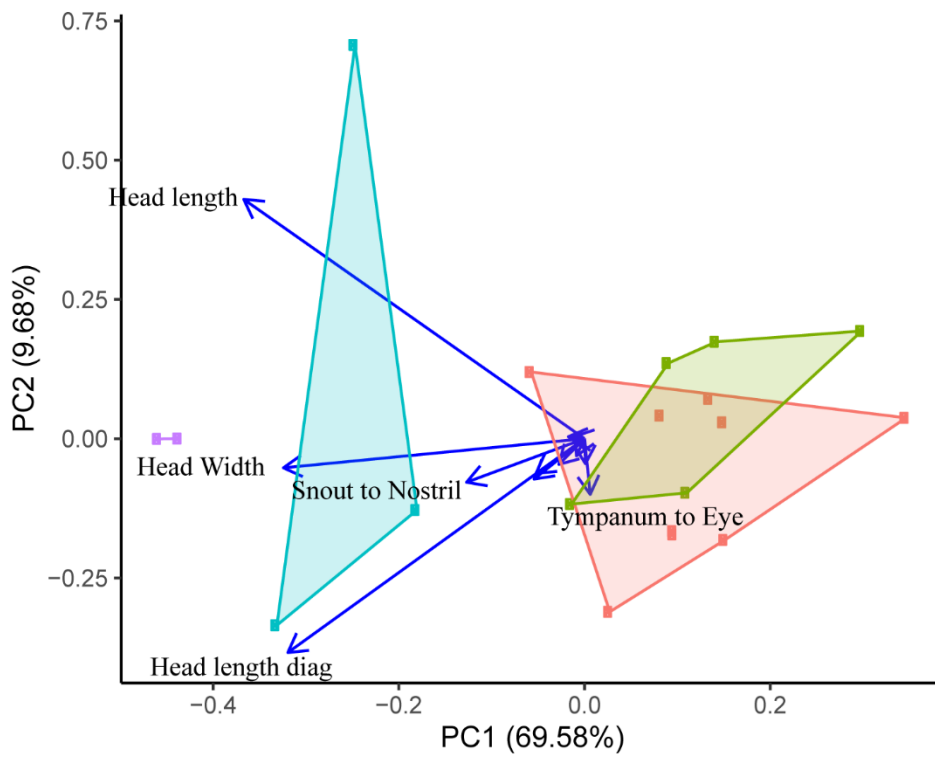
Figure 4.4. PCA plot of samples measured for this study. Blue are males from Sumatra, purple are males from Java, green are females from Sumatra, and red are females from Java. Loadings and Eigenvalues are in Table 4.3.

Table 4.2. Eigenvalues and loadings for figure 4.3. Measurement labels correspond to those listed in Table 4.1.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12
HW	0.0929	0.406258	-0.00753	-0.17833	-0.23503	0.181552	-0.36177	0.335628	-0.4798	-0.46213	0.129463	0.046977
HL	0.284201	0.395346	-0.23912	-0.10011	-0.26395	0.453005	0.218481	0.023027	0.039835	0.594848	0.125117	-0.03871
HLd	0.267753	0.40282	-0.28545	-0.22265	0.130558	-0.5283	-0.11598	0.251338	0.470705	-0.04852	-0.1917	-0.03951
TYM	0.2017	-0.46153	-0.22873	-0.12314	-0.40454	-0.14131	0.4838	0.42609	-0.16494	-0.11916	-0.19225	-0.03493
HWn	0.293958	-0.34546	-0.00299	-0.32398	-0.21804	0.386697	-0.2794	-0.20939	0.519488	-0.30179	0.09649	0.005796
IND	0.310436	0.049938	0.311838	0.498444	0.289193	0.37295	0.088969	0.437526	0.208817	-0.13599	-0.26684	-0.0086
SEM	0.377844	-0.02438	-0.2056	0.086989	0.076098	-0.01199	-0.08385	-0.35798	-0.25486	0.001933	-0.41353	0.65553
ED	0.215963	0.025024	0.525586	-0.65309	0.370167	0.000347	0.282427	-0.00764	-0.16538	0.061389	-0.02831	0.034611
IOD	0.262784	-0.02941	0.561824	0.145297	-0.51307	-0.32823	-0.32068	-0.03226	-0.02852	0.307467	-0.12255	-0.09001
NAE	0.346882	0.176236	0.106529	0.275713	-0.08413	-0.23423	0.409646	-0.24557	0.059527	-0.31428	0.594121	0.126074
SN	0.377625	-0.02802	-0.19492	0.084818	0.179694	-0.00111	-0.0472	-0.34571	-0.29009	-0.10597	-0.17418	-0.72916
TEY	0.306682	-0.38421	-0.16796	0.040101	0.343226	-0.1067	-0.35808	0.312657	-0.1586	0.313292	0.493169	0.082606
EigenValues	27.82401	9.090462	5.746184	4.969604	4.606043	4.019175	3.157125	2.235485	1.666917	1.298118	0.911335	0.415426

Table 4.3. Eigenvalues and loadings for figure 4.4. Measurement labels correspond to those listed in Table 4.1.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12
HW	-0.53675	-0.08669	-0.582	0.101235	0.413653	0.376421	-0.02389	0.121598	-0.15291	0.033164	0.000417	-0.05271
HL	-0.60734	0.71085	0.172236	0.04782	-0.2292	-0.12954	-0.01149	-0.04787	0.12394	-0.02817	0.07736	-0.00014
HLd	-0.52885	-0.63549	0.446508	0.265788	-0.09578	-0.12859	0.076948	-0.04488	0.066299	0.031118	-0.08119	0.029115
TYM	-0.04581	-0.07639	-0.34405	-0.0144	-0.38114	-0.33231	0.36763	0.570822	-0.10171	-0.16004	-0.05542	0.343182
HWn	-0.01818	0.006292	-0.25916	0.026197	-0.27531	-0.23706	0.002259	-0.18994	-0.17097	0.663393	-0.45457	-0.29623
IND	-0.0289	0.00579	-0.07449	-0.1156	-0.01861	0.133899	-0.30726	-0.04464	0.429862	-0.22898	-0.71846	0.334881
SEM	-0.01436	-0.05104	-0.05363	0.010421	-0.5644	0.479779	0.096329	-0.35722	-0.4186	-0.35708	-0.06579	-0.0391
ED	-0.08658	-0.10334	-0.1986	-0.04786	0.026384	-0.54647	-0.60829	-0.21006	-0.33958	-0.31064	0.11545	0.038636
IOD	-0.08989	-0.12003	-0.29468	-0.30763	0.039693	-0.18227	0.398424	-0.61577	0.323918	0.022441	0.223655	0.260531
NAE	-0.21024	-0.12952	0.170058	-0.89409	-0.05275	0.084803	-0.07686	0.219398	-0.09866	0.066768	0.005758	-0.18017
SN	0.001618	-0.07436	-0.03613	0.020976	-0.30024	0.265248	-0.42372	0.056954	0.061651	0.478242	0.366762	0.531423
TEY	0.010162	-0.16559	-0.28597	0.075411	-0.36336	0.029143	-0.20083	0.121323	0.568779	-0.14602	0.243539	-0.54123
EigenValues	9.42456	3.516242	3.230019	3.036241	1.697634	1.129888	1.016556	0.962285	0.609463	0.432352	0.3077	0.230231
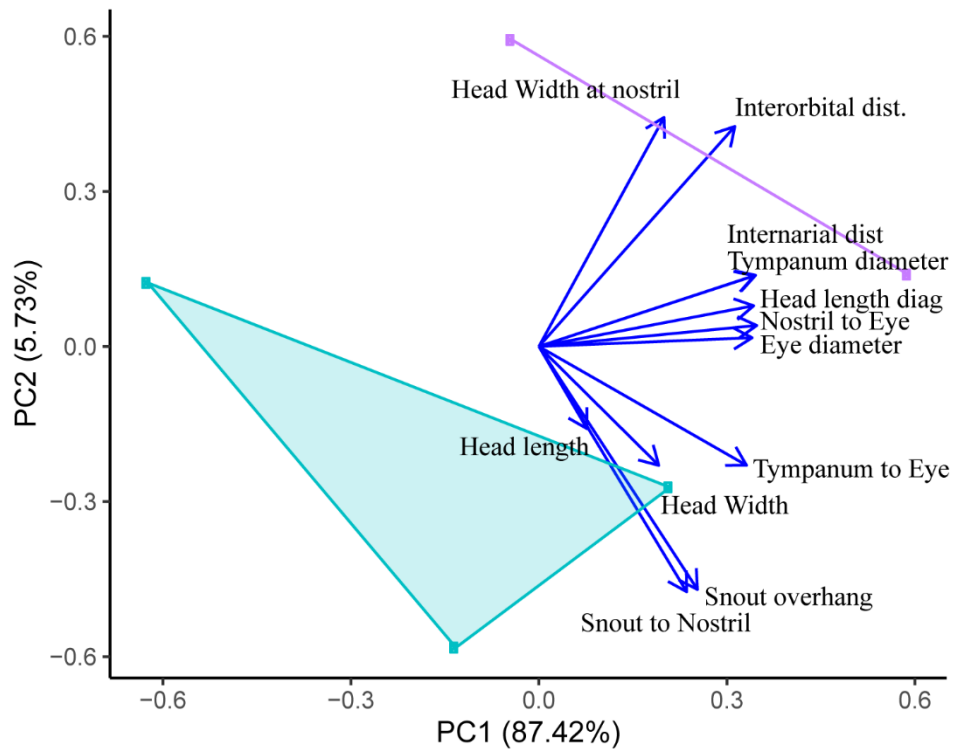
Figure 4.5. PCA plot of male samples measured for this study corrected by total body length (SVL). Blue are males from Sumatra and purple are males from Java. Loadings and Eigenvalues are in Table 4.4.
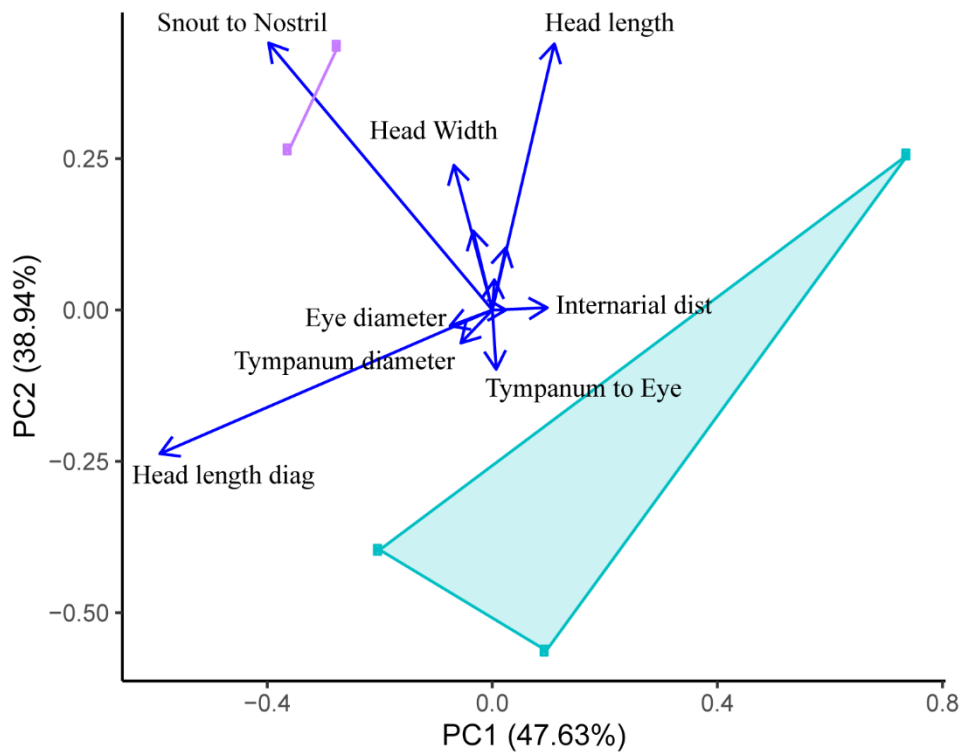
Figure 4.6. PCA plot of male samples measured for this study. Blue are males from Sumatra and purple are males from Java. Loadings and Eigenvalues are in Table 4.5.

	PC1	PC2	PC3	PC4	PC5		PC1	PC2	PC3	PC4	PC5
HW	0.191205	-0.22998	-0.68674	-0.03139	-0.471964727	**HW**	0.191205	-0.22998	-0.68674	-0.03139	-0.47196
HL	0.076719	-0.15895	0.010714	0.691421	-0.128425104	**HL**	0.076719	-0.15895	0.010714	0.691421	-0.12843
HLd	0.342467	0.078747	0.154163	0.147014	0.368795004	**HLd**	0.342467	0.078747	0.154163	0.147014	0.368795
RYM	0.199542	0.443061	-0.55095	0.147994	0.297852763	**RYM**	0.199542	0.443061	-0.55095	0.147994	0.297853
HWn	0.345561	0.137798	0.074641	-0.02705	-0.062553261	**HWn**	0.345561	0.137798	0.074641	-0.02705	-0.06255
IND	0.252844	-0.46988	-0.08431	0.391198	0.284665273	**IND**	0.252844	-0.46988	-0.08431	0.391198	0.284665
SEM	0.340047	0.016934	0.304264	0.008898	-0.35684301	**SEM**	0.340047	0.016934	0.304264	0.008898	-0.35684
ED	0.312483	0.425587	-0.06153	0.001505	0.046415214	**ED**	0.312483	0.425587	-0.06153	0.001505	0.046415
IOD	0.347414	0.040647	0.106649	-0.09698	-0.462317729	**IOD**	0.347414	0.040647	0.106649	-0.09698	-0.46232
NAE	0.235641	-0.47435	-0.16623	-0.52278	0.301357109	**NAE**	0.235641	-0.47435	-0.16623	-0.52278	0.301357
SN	0.3435	0.136725	0.090111	-0.20137	0.113016896	**SN**	0.3435	0.136725	0.090111	-0.20137	0.113017
TEY	0.331089	-0.22978	0.211667	0.018329	0.065307917	**TEY**	0.331089	-0.22978	0.211667	0.018329	0.065308
EigenValues	16.40881	4.200698	3.478531	2.999904	4.84E-16	**EigenValues**	7.035976	6.362429	2.912821	2.339847	3.11E-15

Table 4.4. Eigenvalues and loadings for figure 4.5. Measurement labels correspond to those listed in Table 4.1.

Table 4.5. Eigenvalues and loadings for figure 4.6. Measurement labels correspond to those listed in Table 4.1.

Figure 4.7. PCA plot of female samples measured for this study corrected by total body length (SVL). Green are females from Sumatra and red are females from Java. Loadings and Eigenvalues are in Table 4.6.
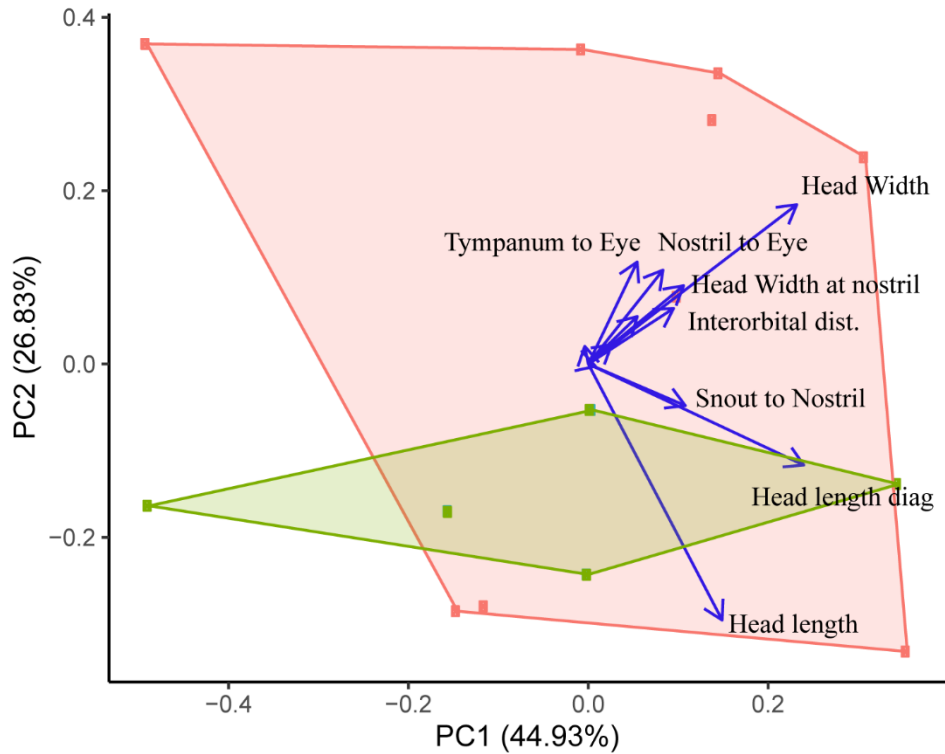
Figure 4.8. PCA plot of female samples measured for this. Green are females from Sumatra and red are females from Java. Loadings and Eigenvalues are in Table 4.7.

Table 4.6. Eigenvalues and loadings for figure 4.7. Measurement labels correspond to those listed in Table 4.1.

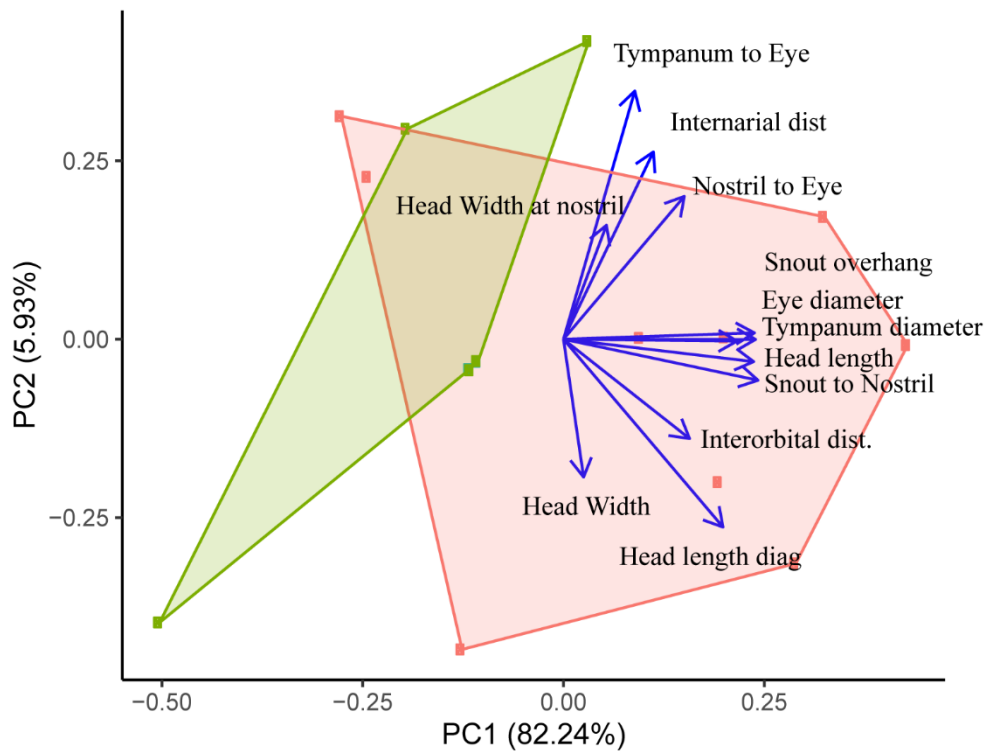|	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12
V1	0.040349	-0.31106	0.07937	-0.03406	0.146625	-0.43274	0.182697	-0.77569	-0.19888	0.017335	-0.01331	-0.10143
V2	0.381312	-0.0506	-0.21452	0.063833	0.026287	0.495344	-0.17089	-0.24306	-0.18126	0.313314	0.575509	-0.07336
V3	0.319729	-0.4238	0.041188	-0.46406	-0.15881	-0.15041	0.188746	0.439155	-0.466	0.04909	0.013402	-0.07106
V4	0.086168	0.257118	-0.0594	-0.62935	-0.45031	-0.1798	-0.11516	-0.19784	0.449551	0.128943	0.136399	-0.04503
V5	0.18018	0.422571	0.328326	-0.30333	0.713989	-0.15809	-0.04999	0.099487	-0.04321	0.128989	0.130779	0.079907
V6	0.347929	-0.00423	-0.07788	0.396903	-0.02856	-0.40949	0.456145	0.216107	0.378737	0.038097	0.380893	0.045736
V7	0.385014	-0.00048	-0.22249	-0.10028	0.147936	0.063835	-0.14566	-0.0628	0.08952	-0.84336	0.010126	-0.14735
V8	0.252759	-0.22419	0.595291	0.24879	-0.19277	-0.1821	-0.62138	0.043782	0.092658	-0.00876	0.043846	0.025584
V9	0.242219	0.32222	0.546169	0.062318	-0.27895	0.356211	0.469619	-0.15207	-0.09812	-0.10913	-0.15184	-0.20582
V10	0.38996	-0.09267	-0.07771	-0.03885	0.005144	0.153546	0.066194	-0.13992	0.104603	0.083062	-0.37442	0.791329
V11	0.382785	0.01427	-0.24261	0.109285	0.139478	-0.02559	-0.11154	0.039689	0.173008	0.365969	-0.56589	-0.51405
V12	0.142882	0.559916	-0.24804	0.210969	-0.28789	-0.36248	-0.18241	-0.02228	-0.544	-0.01818	-0.04631	0.116592
EigenValues	24.94151	6.700175	5.629922	4.993742	3.430109	3.015543	2.542947	2.063457	0.806805	0.671748	0.305336	0.210823

Table 4.7. Eigenvalues and loadings for figure 4.8. Measurement labels correspond to those listed in Table 4.1.

|	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12
V1	0.548329	0.434784	0.261851	0.023097	-0.55518	0.208807	-0.02321	-0.26606	0.072375	0.034204	-0.02308	-0.1054
V2	0.352074	-0.6987	0.056884	0.448624	-0.17791	0.208363	0.213453	0.201751	-0.05378	0.138014	-0.00432	-0.00855
V3	0.566851	-0.27592	0.232804	-0.46836	0.367657	-0.31577	-0.27182	-0.08461	-0.08435	0.041574	-0.05494	-0.03461
V4	0.250574	0.213593	0.149807	0.2152	0.419283	0.26172	-0.01983	0.298578	0.257678	-0.45927	0.45039	0.09394
V5	0.128724	0.129435	-0.04111	0.2042	0.179245	0.002305	0.240239	-0.30837	-0.71059	-0.27008	-0.11874	0.382583
V6	0.061617	0.053468	-0.16409	0.106879	-0.23691	-0.2384	-0.42385	0.156612	-0.10541	0.322827	0.452365	0.563825
V7	0.018893	-0.00637	0.025061	0.540474	0.178957	-0.49254	0.004974	-0.53156	0.378323	0.040887	0.035866	-0.05976
V8	0.224322	0.151762	-0.19632	-0.15872	-0.15426	-0.52346	0.645068	0.349804	0.122317	-0.07143	0.03015	0.075226
V9	0.196372	0.256235	-0.34287	0.048477	0.39481	0.224544	0.191593	-0.03289	-0.12402	0.642003	0.174586	-0.27158
V10	0.255121	-0.11367	-0.81387	-0.06777	-0.08197	0.127226	-0.21908	-0.15732	0.16841	-0.33878	-0.12642	-0.01586
V11	-0.00942	0.048316	-0.06187	0.205249	-0.14879	-0.30794	-0.24177	0.193703	-0.44711	-0.21587	0.255206	-0.65349
V12	0.128414	0.278263	0.001864	0.336215	0.142646	-0.09946	-0.28803	0.452007	0.02379	0.09826	-0.67901	0.065168
EigenValues	4.61347	3.565053	2.471431	1.930633	1.209839	0.882582	0.804427	0.632151	0.399244	0.263572	0.135088	0.080191

Skull Computerized Tomography (CT) Scan Analysis

The micro CT-scans highlighted osteological differences between males and females. The same differences were noted in head width and length between the sexes, but also a much more prominent frontoparietal medial ridge as well as larger odontoids were able to be taken into consideration for landmark placement (Figure 4.9).

Only five males (two from West Sumatra, three from Aceh) and five females (two from West Sumatra, three from Aceh) were able to be scanned for this study. The separation of males from females was very clear across PC1 (Figure 4.10, Table 4.8). The variance explained by PC1 was 66.59% and PC2 explained 10.96% of the variance. The total variance explained by PC1 and PC2 amounts to 77%, with PC1 appearing to be primarily a factor of overall head size. This comparison, while meant to observe differences between males and females, also demonstrated the trend observed in the mensural data with the males separating slightly across PC2, while the females have some overlap across PC1 and PC2.

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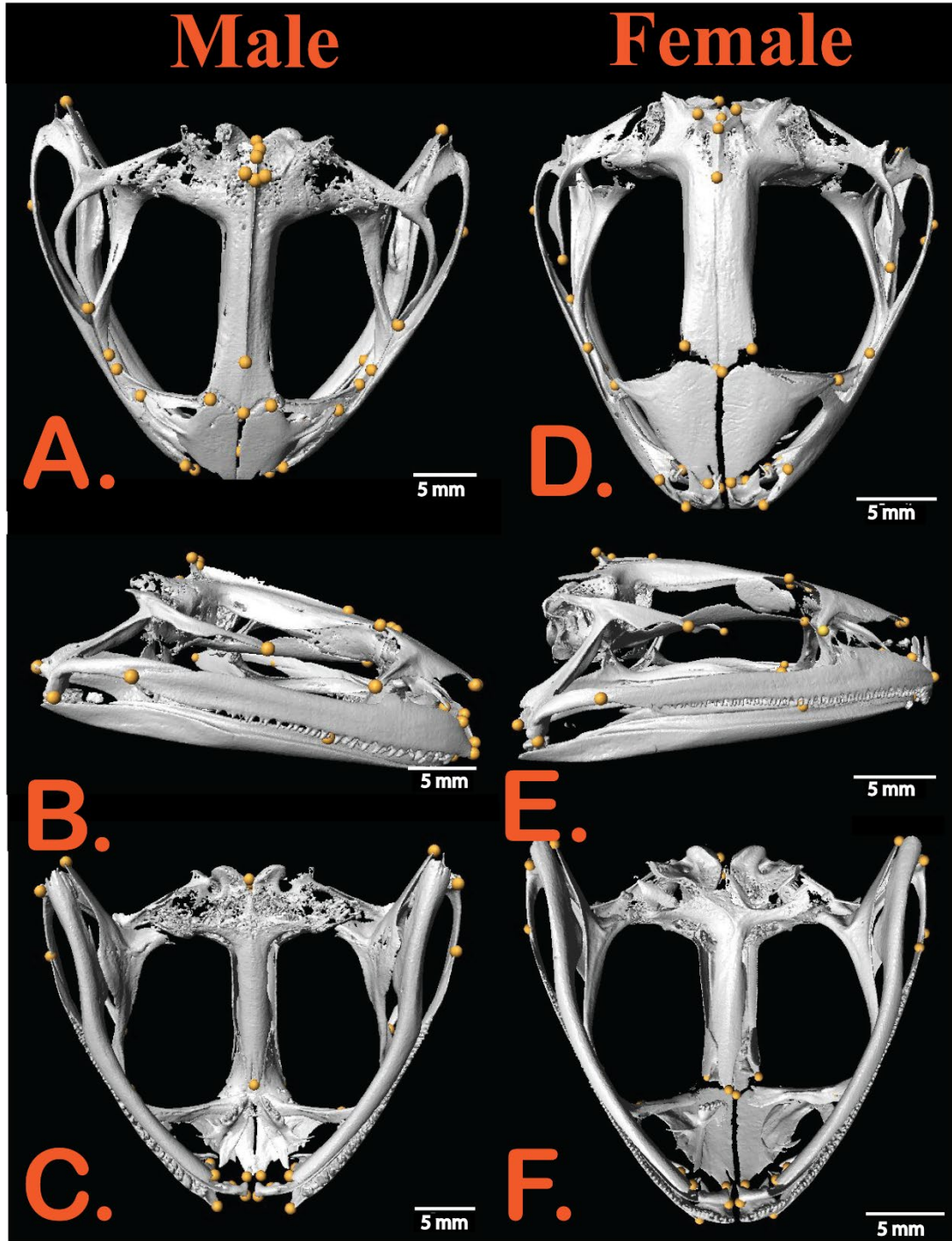
Figure 4.9. CT scans of male (ENS 18805) (A, B, C) and female ENS 19357 (D, E, F) in dorsal (A, D), lateral (B, E) and ventral (C, F) view. Yellow dots represent the landmarks set in Stratovan Checkpoint.
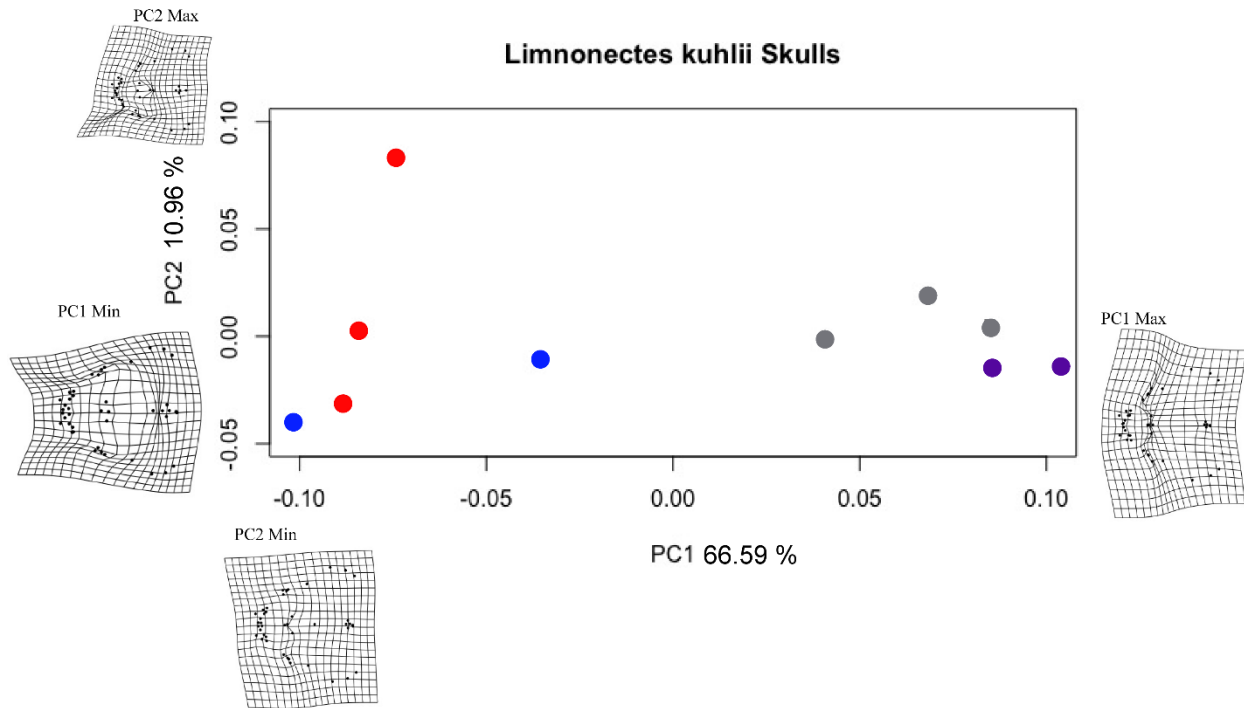
Figure 4.10. Morphometric analysis plot for samples that were micro CT-scanned. Blue dots represent females from West Sumatra and red are females from Aceh. Purple dots represent males from West Sumatra and grey dots are males from Aceh.

Table 4.8. Eigenvalues Figure 4.10.

	Comp1	Comp2	Comp3	Comp4	Comp5	Comp6	Comp7	Comp8	Comp9
Eigenvalues	0.007054	0.001161	0.000719	0.00055	0.000366	0.000283	0.000229	0.000132	1.00E-04
Proportion of Variance	0.665952	0.109568	0.067846	0.051932	0.034574	0.026685	0.021581	0.012421	0.00944
Cumulative Proportion	0.665952	0.77552	0.843366	0.895299	0.929873	0.956558	0.978139	0.99056	1

Discussion

The differences between overall male and female skull morphology were clear, with male skulls demonstrating a larger width, length, as well as other robust characters. These characters, when resulting from sexual selection through male-male combat, are selected for only males of a given group, leading to a distinct set of traits that determine male control over brooding territory. These

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males therefore have access to good brooding localities, more access to females, and more offspring. As populations become isolated over time due to biogeographic events, a distinct set of traits will set in, differentiating males of different groups faster than females, since certain traits of the skull lead to more mating success.

The PCAs conducted for size corrected data all corresponded with traits that associate with head size. Differences in the width at nostril, eye-tympanum distance, and internarial distance all act on the diversity between males and females across X and Y axes. The same measurements also were strong regarding differences between males across the X and Y axes. Females did not demonstrate any difference between Javan and Sumatran groups with the Javan females demonstrating more variance across PC1 than the Sumatran females.

When size corrected there are several traits that influence differences between males and females, as well as, males. However, size is biologically relevant in the *L. kuhlii* complex and an important factor for looking at species groups (Bendik et al., 2013). The uncorrected data demonstrates that overall skull size (HW, HL, HLd) is a very important delimiter between males and females, and males of different species. This male-female difference, in particular, shows how relevant skull size is between the sexes, as skull size is a known influence on mating success for males but a trait that is not advantageous to females. If the trait were advantageous to females as well, we would see some morphological evidence of larger or smaller heads in females as well.

The CT-scanned principal component analysis was completely free from the influence of overall body size on skull morphology and correlates with divisions between groups that were not adjusted for size. The overall skull size indicators (HW, HL, HLd) were primary dividers along PC1, for males to females. In males to males the length of the snout to nostril also acts as a

strong influence across X and Y axes separating the Sumatran males from the Javan males. The snout to nostril difference suggests that it may be a key character in defining species groups.

The range of intrasexual morphological differences among females and males in the *Limnonectes kuhlii* complex are starkly different. While males are separated by differences in skull size and traits such as length of the snout to the nostril, females are morphologically similar to the point that morphological characters alone cannot distinguish populations. Generally in frogs, females are only selected for size (Kupfer, 2007; Shine, 1979), to allow for carrying more eggs. This would suggest that all female traits between species would be similar, not being evolutionarily advantageous for females to have larger skulls. However, in *L. kuhlii*, we see that while females appear to have undergone stabilizing selection, they are on average significantly smaller than males of the same species. Females in the *L. kuhlii* complex select a site based on brooding habitat and not any advertisement of the male, and mate selection through vocalization is reduced in the *L. kuhlii* complex. Females of other Anurans have shown that female body size does not impact female mate choice (Jones et al., 2011), therefore brooding habitat is likely the most important factor in *L. kuhlii* female mate choice.

Male morphology is selected upon by male-male combat (Tsuji and Matsui, 2002) and, therefore, selecting for specific traits that will lend themselves to victory and access to preferred brooding habitats. The selection between males would lead to a specific suite of traits being more prominent in males of differing populations, particularly in the skull, where the greatest influence upon success lies. This is reflected in other intrasexual selection relationships where male-male combat can exacerbate traits such as tusks, odontoids, and head size (Shine, 1979), canines in raccoons (Ritke, 1990), and tufts of bristles in some wolf spider species (Scheffer et al., 1996). In many cases, there is no other evolutionary advantage to these traits aside from

winning territory opportunity for mating. As there are no direct observations of males within the *Limnonectes kuhlii* complex fighting other species or creatures and there are no diet differences determined (Emerson, 1994) between males and females, the morphological trait of big-headedness only lends itself to male-male combat. Such intrasexual selection would act relatively quickly upon populations (Graves, 2010).

Since there is such a strong selection for certain traits (large head) in males, other traits that would be more prominent in certain populations may be passed down as a result, leading to distinct characters between even closely related species. Other characters certainly have an influence in the result of male-male competition (Tinghitella et al., 2018), but being that the skull dimensions of *Limnonectes kuhlii* were so distinct between the sexes, it made sense to target the skull. It should also be noted that multiple measurements across the skull, including some specific structures of the osteo regions of the skull such as the frontoparietal medial ridge and odontoid size, in the micro CT-scans demonstrated a similar trend as the typical morphological measurements. Results from the placement of landmarks on 3-demensional images such as those generated from micro CT-scans, have the ability to accurately collect data across skeletal structures.

Indonesia's geologic history would increase the likelihood of these changes because it contains many young islands (Hall, 2012), which would increase isolation of closely related species. Other disturbances, such as volcanic activity (Wilting et al., 2012, Barber et al. 2005), would also create opportunities for recent speciation. It is expected that males will morphologically be able to be identified as separate groups, while females will show less or no morphological separation between closely related species.

The age of the islands would lead species divergences to also be recent. Continuous uplifting events would isolate populations, genetically isolating them from each other (Heads, 2019; Liew et al., 2020), morphological divergence would follow (Hu et al., 2019). Very little difference in morphology is often associated with recent divergence of species (Ferval et al., 2013). As cryptic species are difficult to uncover determining a number of differences becomes increasingly important. Historical biogeography is often used to relate species models to their given lineages to correlate the idea of dispersal or isolation (Chan et al., 2020). Utilizing morphological methodologies, particularly those that allow for large data collection over single samples such as what can be collected from CT-scan datasets will assist in the morphological uncovering of cryptic species just as increased DNA sequencing techniques opened the door to uncovering cryptic species from genetic divergences (Bickford et al., 2007).

Morphology will remain fundamental in defining species boundaries. Physical traits represent a direct interface between an individual and its habitat and are indispensable in characterizing a species niche. This study demonstrated that skull morphology, coupled with genetics, can be used as evidence to demonstrate differences in closely related species. More direct life history observations of male-male combat would further lend support to the necessity of a larger head size and its influence on mating success.

Chapter 5: Conclusions on the *Limnonectes kuhlii* Complex (Anura: Dicroglossidae) on the Islands of Sumatra and Java, Indonesia

As a highlighted biogeographical hotspot (Myers et al. 2000, Mittermeier et al. 2011) the lack of amphibian diversity noted from Indonesia is often surprising to those unfamiliar with the region. Indonesia has long been a black hole of data due to a myriad of difficulties, including transport. Phylogenetic information fills in large gaps of knowledge for the distribution of species of *Limnonectes*. Working on a project regarding any part of the *L. kuhlii* complex has been described as a daunting task that is only worthy of those who committed egregious acts in a past life (Inger, 2010). The high level of diversity does reflect in other works of uncovered lineages in Southeast Asia, such as those in Thailand (Matsui et al., 2010; McLeod, 2008; Yodthong et al., 2021). Elucidating candidate species is only the first step of many in uncovering species diversity in Indonesia.

This study on the cryptic species within the *Limnonectes kuhlii* complex uncovered multiple clades that require deeper exploration. Species delimitation analyses suggest that up to 24 species within the complex may be present. Divergence data compared to biogeographical events further support the possibility of a high number of species. The connection of divergence dating to geologic and climatic events helped us solidify these candidate species. The biogeographic history discussed here for species in the *Limnonectes kuhlii* complex provides a wealth of knowledge and understanding to how hyper diversification is supported within Indonesia. Previously identified areas of hyper diversity including speciation and endemism included Indochina and Northern Borneo (de Bruyn et al., 2014). Sumatra was highlighted recently at a colleague's defense as an area of high diversification for a group of toads (Sarker,

2020); this study lends further support for Sumatra's high diversity that, prior to these studies, had been overlooked. Further analyses would be needed in order to suggest Sumatra as an area of endemism for *Limnonectes* species, particularly the *L. kuhlii* complex.

The morphological component of this study was meant to determine suites of characteristics that might help morphologically differentiate species from one another. Two distinct methodologies leading to the same result were used utilizing distinct skull characteristics. Both mensural traits as well as morphometric landmarks can be used independently or in tandem to distinguish males as morphologically distinct from one another. In sexual dimorphic comparisons, only one trait is typically observed, leaving many possibly discrete traits yet to be uncovered. By focusing on several skull measurements as well as other osteological characters for morphometric analysis, such as the odontids and frontoparietal medial ridge, more often overlooked traits were used in this work. Further study of sexually dimorphic traits would lead to stronger understanding of species and their history.

The contributions of the collections made should not be discounted but it should be noted that areas of Sumatra and areas of Java remain unsampled, particularly the lowlands. An even larger absence of collections remains for Southeast Asian herpetofauna studies focusing on the areas of Kalimantan and Papua. The diversity of each area has been easier to research on the other sides of these islands (Malaysia and Brunei, Papua New Guinea) and those contributions are enormous; it remains a disservice to species and science alike that many important areas remain unsampled.

The diversity of Indonesia is fascinating from the standpoint of someone studying herpetofaunal diversity, but the greatest contributions are to the species diversity and conservation assessments of the region. As more species, particularly those considered cryptic,

are uncovered, we researchers need to make sure to identify new species ranges for both the new species and the species it was split from. This will assist conservation assessments and, as we learn more about species with limited distributions, we can make greater assessments on what land might be best preserved for conservation or used for harvesting and agriculture.

Appendix I

Appendix I. Samples used in the entire study in different sections.

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
Outgroups							
Occidozyga laevis	SBE 072'	Malaysia: Selangor Dist.			N/A	U66138(12S) U66139(16S)	Emerson et al. 2000
Fejervarya vittigera	PNM 7826	Philippines: Luzon Island: Quezon Provence	14.050	121.541	N/A	AY313683	Evans et al. 2003
F. limnocharis	FMNH 267579	China: Sichuan			N/A	AF261244(12S) AF261262(16S)	Evans et al. 2003
Hoplobatrachus rugulosus	PNM 7827	Philippines: Luzon Island: Laguna Provence	14.155	121.235	N/A	AY313685	Evans et al. 2003
Paa robertingeri	SCUM0405169	China: Sichuan			N/A	DQ458244	Che et al. 2007
P. boulengeri	SCUM37989	China: Sichuan			N/A	DQ458243	Che et al. 2007
Limnonectes							
L. acanthi	TNHC 54922	Philippines: Mindoro Island: Oriental Mindoro Provence	13.438	121.067	N/A	U66120(12S) U66121(16S)	Evans et al. 2003
L. acanthi	PNM 7604	Philippines: Palawan Island: Palawan Provence	9.806	118.686	N/A	AY313722	Evans et al. 2003
L. arathooni	TNHC 59087	Indonesia: Sulawesi: Sulawesi Selatan			N/A	AY313744	Evans et al. 2003
L. blythii	SBE 062'	Malaysia: Endau Rompin National Park			N/A	U55263(12S) U55270(16S)	Evans et al. 2003
L. finchi	FMNH242870	Malaysia: Borneo: Sabah			N/A	U55264(12S) U55271(16S)	Evans et al. 2003

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. grunniens		Indonesia: Haruku Island			N/A	U66124(12S) U66125(16S)	Evans et al. 2003
L. gyldenstolpei	PWRC002	Thailand: Loei			N/A	AF183123(12S) AF183124(16S)	Evans et al. 2003
L. modestus	TNHC 59710	Indonesia: Sulawesi: Sulawesi Utara			N/A	AY313749	Evans et al. 2003
L. ibanorum	FMNH 251721	Indonesia: Borneo: Kalimantan Barat			N/A	U66122(12S) U66123(16S)	Evans et al. 2003
L. ingeri	FMNH 251722	Malaysia: Borneo: Sarawak			N/A	U55268(12S) U55275(16S)	Evans et al. 2003
L. kardasani	LSUMZ 81722	Indonesia: Lombok			N/A	AY313693	Evans et al. 2003
L. laticeps	SBE 071'	Malaysia: Selangor District			N/A	AF183125(12S) AF183126(16S)	Evans et al. 2003
L. leporinus	AMNH 167165	Indonesia: Borneo: Kalimantan Timor	0.532	117.465	N/A	AY313691	Evans et al. 2003
L. leporinus	FMNH 230212	Malaysia: Borneo: Sabah			N/A	U55262(12S) U55269(16S)	Evans et al. 2003
L. leytensis	USNM 222546	Philippines: Samar Island: Samar	11.437	124.367	N/A	AF183129(12S) AF183130(16S)	Evans et al. 2003
L. macrocephalus	FSO 54563	Philippines: Luzon Island: Cagayan	17.500	121.750	N/A	U66116(12S) U66117(16S)	Evans et al. 2003
L. macrodon	FMNH 257159	Indonesia: Java: Java Barat			N/A	U66132(12S) U66133(16S)	Evans et al. 2003
L. magnus	USNM 534311	Philippines: Samar Island: Samar	11.437	124.367	N/A	U66118(12S) U66119(16S)	Evans et al. 2003
L. microdiscus	LSUMZ 81739	Indonesia: Java: Sukabumi			N/A	AY313688	Evans et al. 2003
L. microtympanum	AMNH 16176??	Indonesia: Sulawesi: Sulawesi Selatan	4.494	119.767	N/A	AY313743	Evans et al. 2003

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. palavensis	FMNH 233080	Malaysia: Borneo: Sabah			N/A	U55266(12S) U55273(16S)	Evans et al. 2003
L. paramacrodon	FMNH 248283	Brunei: Totong			N/A	U55267(12S) U55274(16S)	Evans et al. 2003
L. parvus	PNM 7447	Philippines: Mindanao Island: Davao del Norte	7.186	125.416	N/A	AY313694	Evans et al. 2003
Limnonectes kuhlii Complex							
L. kuhlii 1	MZBamph6501	Indonesia: Java: Sukabumi	-6.924	106.922	N/A	AY313687	Evans et al. 2003
L. kuhlii 1	MZB	Indonesia: Java: Cibodas: Mt Gede	-6.780	106.947	N/A	AF183137(12S) AF183138(16S)	Evans et al. 2003
L. kuhlii 2	RMBR 515	Indonesia: Sumatra: Batu Layang	-3.464	102.316	N/A	HM067245	McLeod 2010
L. kuhlii 2	RMBR 393	Indonesia: Sumatra: Batu Layang	-3.464	102320.000	N/A	HM067244	McLeod 2010
L. fragilis	SCUMH008	China: Hainan Island: Mt. Limu	19.135	109.733	N/A	DQ458235	Che et al. 2007
L. kuhlii 4	FMNH 262722	Cambodia: Ratanakiri	14.188	107.293	N/A	HM067166	McLeod 2010
L. kuhlii 4	FMNH 262723	Cambodia: Ratanakiri	14.188	107.293	N/A	HM067167	McLeod 2010
L. kuhlii 4	FMNH 262724	Cambodia: Ratanakiri	14.188	107.293	N/A	HM067168	McLeod 2010
L. kuhlii 4	FMNH 262725	Cambodia: Ratanakiri	14.188	107.293	N/A	HM067169	McLeod 2010
L. kuhlii 4	FMNH 262726	Cambodia: Stung Treng	14.268	106.629	N/A	HM067170	McLeod 2010
L. kuhlii 4	FMNH 262727	Cambodia: Stung Treng	14.268	106.629	N/A	HM067171	McLeod 2010

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. kuhlii 4	FMNH 262728	Cambodia: Stung Treng	14.268	106.629	N/A	HM067172	McLeod 2010
L. kuhlii 4	FMNH 262729	Cambodia: Stung Treng	14.268	106.629	N/A	HM067173	McLeod 2010
L. kuhlii 4	FMNH 262730	Cambodia: Stung Treng	14.268	106.629	N/A	HM067174	McLeod 2010
L. kuhlii 5	FMNH 258505	Lao PDR: Xe Kong Provence	16.009	106.917	N/A	HM067146	McLeod 2010
L. kuhlii 5	FMNH 258506	Lao PDR: Xe Kong Provence	16.009	106.917	N/A	HM067147	McLeod 2010
L. kuhlii 5	FMNH 258507	Lao PDR: Xe Kong Provence	16.009	106.917	N/A	HM067148	McLeod 2010
L. kuhlii 5	FMNH 258508	Lao PDR: Xe Kong Provence	16.009	106.925	N/A	HM067149	McLeod 2010
L. kuhlii 5	FMNH 258509	Lao PDR: Xe Kong Provence	16.009	106.925	N/A	HM067150	McLeod 2010
L. kuhlii 5	FMNH 258510	Lao PDR: Xe Kong Provence	16.009	106.925	N/A	HM067151	McLeod 2010
L. kuhlii 5	FMNH 258511	Lao PDR: Xe Kong Provence	16.009	106.925	N/A	HM067152	McLeod 2010
L. kuhlii 5	FMNH 258512	Lao PDR: Xe Kong Provence	16.069	106.975	N/A	HM067153	McLeod 2010
L. kuhlii 5	FMNH 258513	Lao PDR: Xe Kong Provence	16.069	106.975	N/A	HM067154	McLeod 2010
L. kuhlii 5	FMNH 258514	Lao PDR: Xe Kong Provence	16.069	106.975	N/A	HM067155	McLeod 2010
L. kuhlii 6	FRIM 1141	Malaysia: Pahang	3.423	101.786	N/A	HM067200	McLeod 2010
L. kuhlii 6	LSUHC7034	Malaysia: Perak	5.569	101.655	N/A	HM067230	McLeod 2010

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. kuhlii 6	LSUHC5008	Malaysia: Pahang	3.087	103.050	N/A	HM067229	McLeod 2010
L. kuhlii 6	LSUHC4922	Malaysia: Pahang	3.087	103.050	N/A	HM067228	McLeod 2010
L. fujianensis	NMNST 16602	Taiwan ROC	24.784	121.281	N/A	HM067231	McLeod 2010
L. fujianensis	NMNST 16603	Taiwan ROC	24.784	121.281	N/A	HM067232	McLeod 2010
L. fujianensis	NMNST 16604	Taiwan ROC	24.784	121.281	N/A	HM067233	McLeod 2010
L. fujianensis	NMNST 16605	Taiwan ROC	24.784	121.281	N/A	HM067234	McLeod 2010
L. fujianensis	NMNST 16606	Taiwan ROC	24.784	121.281	N/A	HM067235	McLeod 2010
L. fujianensis	NMNST 16650	Taiwan ROC	23.923	120.890	N/A	HM067236	McLeod 2010
L. fujianensis	NMNST 16651	Taiwan ROC	23.923	120.890	N/A	HM067237	McLeod 2010
L. fujianensis	NMNST 16652	Taiwan ROC	23.923	120.890	N/A	HM067238	McLeod 2010
L. fujianensis	NMNST 16653	Taiwan ROC	23.923	120.890	N/A	HM067239	McLeod 2010
L. fujianensis	NMNST 16654	Taiwan ROC	23.923	120.890	N/A	HM067240	McLeod 2010
L. fujianensis	KIZ YP027	China			N/A	DQ118518(12S) DQ118474(16S)	McLeod 2010
L. fujianensis		China: Anhui			N/A	NC007440	McLeod 2010
L. fujianensis	YNUHU20026017	China: Fujian	27.533	117.400	N/A	DQ458234	Che et al. 2007

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. fujianensis	FMNH 257133	Taiwan ROC			N/A	AF183131(12S) AF183132(16S)	Emerson et al. 2000
L. bannaensis	TAO 693	Vietnam: Ha Giang	22.774	104.867	N/A	HM067246	McLeod 2010
L. bannaensis	TAO 694	Vietnam: Ha Giang	22.774	104.867	N/A	HM067247	McLeod 2010
L. bannaensis	AMNH 106382	Vietnam: Quang Binh Provence	17.687	105.750	N/A	HM067269	McLeod 2010
L. bannaensis	AMNH 106383	Vietnam: Quang Binh Provence	17.687	105.750	N/A	HM067270	McLeod 2010
L. bannaensis	TNE-05	Vietnam: Ha Giang District	22.652	105.317	N/A	HM067261	McLeod 2010
L. bannaensis	TNE-06	Vietnam: Ha Giang District	22.773	104.882	N/A	HM067262	McLeod 2010
L. bannaensis	FMNH 255140	Lao PDR: Huaphahn Provence	20.233	103.267	N/A	HM067133	McLeod 2010
L. bannaensis	FMNH 255141	Lao PDR: Huaphahn Provence	20.233	103.267	N/A	HM067134	McLeod 2010
L. kuhlii 9	CAS 205260	Myanmar: Sagaing Division	22.300	94.414	N/A	HM067285	McLeod 2010
L. kuhlii 9	CAS 205261	Myanmar: Sagaing Division	22.300	94.414	N/A	HM067286	McLeod 2010
L. kuhlii 9	CAS 216154	Myanmar: Mandalay Division	22.912	96.101	N/A	HM067303	McLeod 2010
L. kuhlii 9	CAS 219994	Myanmar: Chin State	21.372	93.979	N/A	HM067304	McLeod 2010
L. kuhlii 9	CAS 221808	Myanmar: Shan State	23.090	96.250	N/A	HM067308	McLeod 2010
L. kuhlii 9	CAS 224555	Myanmar: Kachin State	27.491	97.836	N/A	HM067309	McLeod 2010

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. kuhlii 9	CAS 224593	Myanmar: Kachin State	27.509	97.834	N/A	HM067310	McLeod 2010
L. megastomias	FMNH 266220	Thailand: Sa Kaeo Province	14.106	102.256	N/A	HM067183	McLeod 2010
L. megastomias	FMNH 266221	Thailand: Sa Kaeo Province	14.106	102.256	N/A	HM067184	McLeod 2010
L. megastomias	KU 307760	Thailand: Nakhon Ratchasima Province	14.494	101.883	N/A	HM067201	McLeod 2010
L. megastomias	KU 307761	Thailand: Nakhon Ratchasima Province	14.494	101.883	N/A	HM067202	McLeod 2010
L. megastomias	KU 307762	Thailand: Nakhon Ratchasima Province	14.494	101.883	N/A	HM067203	McLeod 2010
L. kuhlii 11	FMNH 266212	Thailand: Loei Provence	17.334	101.500	N/A	HM067175	McLeod 2010
L. kuhlii 11	FMNH 266213	Thailand: Loei Provence	17.280	101.517	N/A	HM067176	McLeod 2010
L. kuhlii 11	FMNH 266214	Thailand: Loei Provence	17.280	101.517	N/A	HM067177	McLeod 2010
L. kuhlii 11	FMNH 266215	Thailand: Loei Provence	17.280	101.526	N/A	HM067178	McLeod 2010
L. kuhlii 11	FMNH 266216	Thailand: Loei Provence	17.259	101.502	N/A	HM067179	McLeod 2010
L. kuhlii 11	FMNH 266217	Thailand: Loei Provence	17.259	101.502	N/A	HM067180	McLeod 2010
L. kuhlii 11	FMNH 266218	Thailand: Loei Provence	17.259	101.502	N/A	HM067181	McLeod 2010
L. kuhlii 11	FMNH 266219	Thailand: Loei Provence	17.259	101.506	N/A	HM067182	McLeod 2010
L. kuhlii 12	CAS 221714	Myanmar: Shan State	20.711	96.487	N/A	HM067306	McLeod 2010

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. kuhlii 12	CAS 230947	Myanmar: Shan State	20.692	96.506	N/A	HM067315	McLeod 2010
L. kuhlii 12	CAS 230948	Myanmar: Shan State	20.692	96.506	N/A	HM067316	McLeod 2010
L. kuhlii 12	CAS 230949	Myanmar: Shan State	20.692	96.506	N/A	HM067317	McLeod 2010
L. kuhlii 12	FMNH 258517	Lao PDR: Phongsaly Province	22.094	102.213	N/A	HM067156	McLeod 2010
L. kuhlii 12	FMNH 258518	Lao PDR: Phongsaly Province	22.094	102.213	N/A	HM067157	McLeod 2010
L. kuhlii 12	FMNH 258521	Lao PDR: Phongsaly Province	22.094	102.214	N/A	HM067160	McLeod 2010
L. kuhlii 12	CUMZA 2003.5	Thailand: Chiang Mai	18.837	98.902	N/A	HM067353	McLeod 2010
L. kuhlii 12	CUMZA 2003.8	Thailand: Chiang Mai	18.837	98.902	N/A	HM067352	McLeod 2010
L. kuhlii 13	AMNH 106355	Vietnam: Ha Giang Provence	22.761	104.882	N/A	HM067267	McLeod 2010
L. kuhlii 13	TNE-02	Vietnam: Ha Giang Provence	22.771	104.850	N/A	HM067258	McLeod 2010
L. kuhlii 13	TAO 697	Vietnam: Ha Giang Provence	22.774	104.867	N/A	HM067250	McLeod 2010
L. kuhlii 13	TAO 699	Vietnam: Ha Giang Provence	22.774	104.867	N/A	HM067252	McLeod 2010
L. kuhlii 14	FMNH 257155	Malaysia: Borneo: Sabah	6.035	116.547	N/A	HM067144	McLeod 2010
L. kuhlii 14	FMNH 257156	Malaysia: Borneo: Sabah	6.035	116.547	N/A	HM067145	McLeod 2010
L. kuhlii 14	FMNH 234378	Malaysia: Borneo: Sabah	6.035	116.547	N/A	HM067117	McLeod 2010

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. kuhlii 14	FMNH 257154	Malaysia: Borneo: Sabah	6.035	116.547	N/A	HM067143	McLeod 2010
L. kuhlii 14	FMNH 234375	Malaysia: Borneo: Sabah	6.035	116.547	N/A	HM067116	McLeod 2010
L. kuhlii 15	FMNH 235674	Malaysia: Borneo: Sabah	6.300	116.700	N/A	HM067121	McLeod 2010
L. kuhlii 15	FMNH 235677	Malaysia: Borneo: Sabah	6.300	116.700	N/A	HM067122	McLeod 2010
L. kuhlii 16	FMNH 238471	Malaysia: Borneo: Sabah	4.900	115.700	N/A	HM067123	McLeod 2010
L. kuhlii 16	FMNH 238517	Malaysia: Borneo: Sabah	5.217	115.950	N/A	HM067125	McLeod 2010
L. kuhlii 16	FMNH 273417	Malaysia: Borneo: Sabah	1.907	113.090	N/A	HM067197	McLeod 2010
L. kuhlii 16	FMNH 238615	Malaysia: Borneo: Sabah	5.217	115.950	N/A	HM067128	McLeod 2010
L. kuhlii 16	FMNH 243627	Malaysia: Borneo: Sabah	5.217	115.950	N/A	HM067132	McLeod 2010
L. kuhlii 16	FMNH 243619	Malaysia: Borneo: Sabah	5.217	115.950	N/A	HM067131	McLeod 2010
L. kuhlii 16	FMNH 238661	Malaysia: Borneo: Sabah	5.217	115.950	N/A	HM067129	McLeod 2010
L. kuhlii 16	FMNH 238534	Malaysia: Borneo: Sabah	5.217	115.950	N/A	HM067126	McLeod 2010
L. kuhlii 17	FMNH 238511	Malaysia: Borneo: Sabah	4.900	115.700	N/A	HM067124	McLeod 2010
L. kuhlii 17	FMNH 243604	Malaysia: Borneo: Sabah	4.900	115.700	N/A	HM067130	McLeod 2010
L. kuhlii 18	FMNH 273423	Malaysia: Borneo: Sarawk	2.907	113.090	N/A	HM067198	McLeod 2010

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. kuhlii 18	FMNH 273428	Malaysia: Borneo: Sarawk	2.907	113.090	N/A	HM067199	McLeod 2010
L. kuhlii 18	FMNH 273405	Malaysia: Borneo: Sarawk	2.656	112.904	N/A	HM067196	McLeod 2010
L. kuhlii 18	FMNH 273342	Malaysia: Borneo: Sarawk	2.656	112.904	N/A	HM067195	McLeod 2010
L. kuhlii 18	FMNH 273341	Malaysia: Borneo: Sarawk	2.656	112.904	N/A	HM067194	McLeod 2010
L. asperatus	BJE 170	Indonesia: Serasan Island	2.509	109.024	N/A	HM067283	McLeod 2010
L. asperatus	BJE 171	Indonesia: Serasan Island	2.509	109.024	N/A	HM067284	McLeod 2010
L. asperatus	FMNH 252416	Indonesia: Borneo: Kalimantan Selatan	2.217	112.904	N/A	AF183127(12S) AF183128(16S)	Emerson et al. 2000
L. asperatus	RMBR 1193	Indonesia: Borneo: Kalimantan Selatan	-0.604	113.933	N/A	HM067241	McLeod 2010
L. asperatus	RMBR 707	Indonesia: Borneo: Kalimantan Selatan	-0.724	112.281	N/A	HM067242	McLeod 2010
L. asperatus	RMBR761	Indonesia: Borneo: Kalimantan Selatan	-0.724	112.281	N/A	HM067243	McLeod 2010
L. asperatus	LSUHC 4090	Malaysia: Borneo: Sarawk	4.198	114.063	N/A	HM067227	McLeod 2010
L. kuhlii 20	FMNH 234394	Malaysia: Borneo: Sabah	4.900	115.700	N/A	HM067118	McLeod 2010
L. kuhlii 20	LSUHC 4089	Malaysia: Borneo: Sarawak	4.198	114.063	N/A	HM067226	McLeod 2010
L. kuhlii 20	FMNH 248357	Brunei: Belait District			N/A	AF183133(12S) AF183134(16S)	Emerson et al. 2000
L. kuhlii 20	FMNH 234395	Malaysia Borneo: Sabah	4.900	115.700	N/A	HM067119	McLeod 2010

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. kuhlii 20	FMNH 238554	Malaysia Borneo: Sabah	4.900	115.700	N/A	HM067127	McLeod 2010
L. kuhlii 21	FMNH 230306	Malaysia Borneo: Sabah	4.833	117.594	N/A	HM067112	McLeod 2010
L. kuhlii 21	FMNH 230311	Malaysia Borneo: Sabah	4.833	117.584	N/A	HM067113	McLeod 2010
L. kuhlii 21	FMNH 230312	Malaysia Borneo: Sabah	4.833	117.583	N/A	HM067114	McLeod 2010
L. kuhlii 21	FMNH 230313	Malaysia Borneo: Sabah	4.833	117.598	N/A	HM067115	McLeod 2010
L. kuhlii 21	FMNH 235665	Malaysia Borneo: Sabah	6.300	116.700	N/A	HM067120	McLeod 2010
L. kuhlii 21	FMNH 230302	Malaysia Borneo: Sabah	4.833	117.598	N/A	AF183135(12S) AF183136(16S)	Emerson et al. 2000
L. kuhlii 22	AMNH167141	Indonesia: Kalimantan	0.532	117.465	N/A	AY313686	Evans et al. 2003
L. sisikdagu	ENS 15175	Indonesia: Sumatra: Lampung: Mt. Pesarawan	-5.186	105.072	N/A	KY228869	Kurniawan and Fahmi unpublished
L. sisikdagu	ENS 14088	Indonesia: Sumatra: Lampung: Tanggamus	-5.282	104.558	N/A	KY228870	Kurniawan and Fahmi unpublished
L. sisikdagu	MZB 22325 ENS 14519	Indonesia: Sumatra: Lampung: Tanggamus	-5.315	104.530	N/A	KY228871	Kurniawan and Fahmi unpublished
L. sisikdagu	ENS 14866	Indonesia: Sumatra: Lampung: Western Lampung	-4.965	103.895	N/A	KY228872	Kurniawan and Fahmi unpublished
L. sisikdagu	FMNH 266617	Indonesia: Sumatra Barat			N/A	JF836880	McLeod et al. 2011
L. sisikdagu	FMNH 266612	Indonesia: Sumatra Barat			N/A	JF836881	McLeod et al. 2011

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. sisikdagu	ENS 14530	Indonesia: Sumatra: Lampung: Tanggamus	-5.315	104.530	N/A	KY228862	Kurniawan and Fahmi unpublished
L. kuhlii	ENS 15916	Indonesia: Java: Java Barat: Bogor	-6.742	107.006	SAMN24058352	KY228864	Kurniawan and Fahmi unpublished ddRADs: this paper
L. kuhlii	ENS 15052	Indonesia: Java: Banten	-6.331	105.969	SAMN24058333	KY228863	Kurniawan and Fahmi unpublished ddRADs: this paper
L. sisikdagu	ENS 16573	Indonesia: Sumatra: Sumatra Utara: Toba	2.616	99.051	N/A	KY132184	Kurniawan and Fahmi unpublished
L. kuhlii	ENS 16229	Indonesia: Java: Java Barat: Bandung	-7.294	107.635	N/A	KY228865	Kurniawan and Fahmi unpublished
L. sisikdagu	ENS 15383	Indonesia: Sumatra: Sumatra Utara: Rambung Baru	3.377	98.598	N/A	KY132180	Kurniawan and Fahmi unpublished
L. sisikdagu	ENS 16939	Indonesia: Sumatra: Sumatra Utara: Rau Batang Gadis	0.709	99.517	N/A	KY228867	Kurniawan and Fahmi unpublished
L. sisikdagu	FMNH 266637	Indonesia: Sumatra: Sumatra Barat			N/A	JF836879	McLeod et al. 2011
L. sisikdagu	FMNH 266619	Indonesia: Sumatra: Sumatra Barat			N/A	JF836877	McLeod et al. 2011
L. sisikdagu	FMNH 266621	Indonesia: Sumatra: Sumatra Barat			N/A	JF836876	McLeod et al. 2011
L. sisikdagu	FMNH 266618	Indonesia: Sumatra: Sumatra Barat			N/A	JF836875	McLeod et al. 2011
L. sisikdagu	FMNH 266615	Indonesia: Sumatra: Sumatra Barat			N/A	JF836874	McLeod et al. 2011
L. sisikdagu	ENS 16060	Indonesia: Sumatra: Sumatra Barat: Solok	-1.136	100.803	SAMN24058360	KY132182	Kurniawan and Fahmi unpublished ddRADs: this paper
L. sisikdagu	FMNH 266616	Indonesia: Sumatra: Sumatra Barat			N/A	JF836878	McLeod et al. 2011

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. sisikdagu	FMNH 266610	Indonesia: Sumatra: Sumatra Barat			N/A	JF836873	McLeod et al. 2011
L. sisikdagu	ENS 14432	Indonesia: Sumatra: Sumatra Barat: Sumber Tapan	-2.043	101.311	SAMN24058325	KY132179	Kurniawan and Fahmi unpublished ddRADs: this paper
L. sisikdagu	ENS 19451	Indonesia: Sumatra: Aceh: Bener Merah	4.715	96.778	N/A	KY228868	Kurniawan and Fahmi unpublished
L. sisikdagu	ENS 19452	Indonesia: Sumatra: Aceh: Bener Merah	4.715	96.778	SAMN24058392	KY228866	Kurniawan and Fahmi unpublished ddRADs: this paper
L. sisikdagu	ENS 16566	Indonesia: Sumatra: Sumatra Utara: Bandar Baru	3.267	98.539	N/A	KY132183	Kurniawan and Fahmi unpublished
L. sisikdagu	ENS 15729	Indonesia: Sumatra: Sumatra Utara: Labuhan Batu	2.328	99.719	SAMN24058342	KY132181	Kurniawan and Fahmi unpublished ddRADs: this paper
L. kuhlii	ENS 19858	Indonesia: Java: Java Timur: Magetan	-7.677	111.203	SAMN24058400	Pending(12S) OL897187(16S)	this paper
L. kuhlii	ENS 19859	Indonesia: Java: Java Timur: Magetan	-7.677	111.204	SAMN24058401	Pending(12S) OL897189(16S)	this paper
L. kuhlii	NK 0275	Indonesia: Java: Java Tengah: Wonosobo	-7.347	109.918	SAMN24058435	Pending(12S OL897194(16S)	this paper
L. kuhlii	NK 0276	Indonesia: Java: Java Tengah: Wonosobo	-7.347	109.918	SAMN24058436	Pending(12S) OL897196(16S)	this paper
L. sisikdagu	ENS 20044	Indonesia: Sumatra: Aceh: Gayo Lues	3.982	96.864	SAMN24058409	Pending(12S) OL897216(16S)	this paper
L. kuhlii	MZB 22305 ENS 15915	Indonesia: Java: Java Barat: Kabupaten Cianjur	-6.742	107.006	SAMN24058351	Pending(12S) OL897200(16S)	this paper
L. sisikdagu	ENS 18744	Indonesia: Sumatra: Bengkulu: Kapahiang City	-3.659	102.558	SAMN24058383	Pending(12S) OL897228(16S)	this paper

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. sisikdagu	ENS 19132	Indonesia: Sumatra: Sumatra Barat: Bukittinggi	-0.318	100.306	SAMN24058389	Pending(12S) OL897214(16S)	this paper
L. sisikdagu	MZB 22290 ENS 16004	Indonesia: Sumatra: Jambi: Kabupaten Kurinci	-1.712	101.369	SAMN24058358	Pending(12S) OL897215(16S)	this paper
L. sisikdagu	ENS 15548	Indonesia: Sumatra: Sumatra Utara: Samosir	2.619	98.804	SAMN24058339	Pending(12S) OL897225(16S)	this paper
L. sisikdagu	ENS 20063	Indonesia: Sumatra: Aceh: Gayo Lues	3.983	96.864	SAMN24058415	Pending(12S) OL897222(16S)	this paper
L. sisikdagu	ENS 20065	Indonesia: Sumatra: Aceh: Gayo Lues	3.983	96.864	SAMN24058416	Pending(12S) OL897223(16S)	this paper
L. sisikdagu	ENS 20047	Indonesia: Sumatra: Aceh: Gayo Lues	3.982	96.864	SAMN24058412	Pending(12S) OL897224(16S)	this paper
L. sisikdagu	ENS 20004	Indonesia: Sumatra: Aceh: Gayo Lues	3.982	96.864	SAMN24058408	Pending(12S) OL897221(16S)	this paper
L. sisikdagu	ENS 21231	Indonesia: Sumatra: Aceh: Gayo Lues	4.126	97.592	SAMN24058424	Pending(12S) OL897227(16S)	this paper
L. sisikdagu	ENS 16553	Indonesia: Sumatra: Sumatra Utara: Deli Serdang	3.268	98.540	SAMN24058366	Pending(12S) OL897219(16S)	this paper
L. sisikdagu	ENS 15728	Indonesia: Sumatra: Sumatra Utara: North Labuhanbatu	2.328	99.719	SAMN24058341	Pending(12S) OL897226(16S)	this paper
L. sisikdagu	ENS 20045	Indonesia: Sumatra: Aceh: Gayo Lues	3.982	96.864	SAMN24058410	Pending(12S) OL897217(16S)	this paper
L. sisikdagu	ENS 20920	Indonesia: Sumatra: Aceh: Aceh tenggera	3.635	97.721	SAMN24058422	Pending(12S) OL897220(16S)	this paper
L. sisikdagu	ENS 21055	Indonesia: Sumatra: Aceh: Pidie Jaya	4.855	96.218	SAMN24058423	Pending(12S) OL897213(16S)	this paper

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. sisikdagu	ENS 17450	Indonesia: Sumatra: Sumatra Selatan: Pagar Alam City	-4.014	103.188	SAMN24058371	Pending(12S) OL897229(16S)	this paper
L. sisikdagu	ENS 18732	Indonesia: Sumatra: Bengkulu: Kapahiang City	-3.659	102.558	SAMN24058381	Pending(12S) OL897230(16S)	this paper
L. kuhlii	ENS 15911	Indonesia: Java: Java Barat: Kabupaten Cianjur	-6.742	107.006	SAMN24058347	Pending(12S) OL897208(16S)	this paper
L. kuhlii	UTA-A-65791 ENS 15920	Indonesia: Java: Java Barat: Kabupaten Cianjur	-6.742	107.006	SAMN24058356	Pending(12S) OL897206(16S)	this paper
L. kuhlii	ENS 15918	Indonesia: Java: Java Barat: Kabupaten Cianjur	-6.742	107.006	SAMN24058354	Pending(12S) OL897204(16S)	this paper
L. kuhlii	ENS 15919	Indonesia: Java: Java Barat: Kabupaten Cianjur	-6.742	107.006	SAMN24058355	Pending(12S) OL897203(16S)	this paper
L. kuhlii	ENS 15909	Indonesia: Java: Java Barat: Kabupaten Cianjur	-6.742	107.006	SAMN24058345	Pending(12S) OL897201(16S)	this paper
L. kuhlii	ENS 15910	Indonesia: Java: Java Barat: Kabupaten Cianjur	-6.742	107.006	SAMN24058346	Pending(12S) OL897205(16S)	this paper
L. kuhlii	ENS 15913	Indonesia: Java: Java Barat: Kabupaten Cianjur	-6.742	107.006	SAMN24058349	Pending(12S) OL897202(16S)	this paper
L. kuhlii	ENS 15094	Indonesia: Java: Banten: Pandeglang	-6.331	105.969	SAMN24058334	Pending(12S) OL897199(16S)	this paper
L. sisikdagu	ENS 18532	Indonesia: Sumatra: Bengkulu: Bengkulu Tenggah	-3.658	102.556	SAMN24058380	Pending(12S) OL897186(16S)	this paper
L. kuhlii	ENS 16168	Indonesia: Java: Java Barat: Cianjur	-7.247	107.357	SAMN24058363	Pending(12S) OL897197(16S)	this paper

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. kuhlii	NK 0291	Indonesia: Java: Java Tengah: Wonosobo	-7.346	109.918	SAMN24058439	Pending(12S) OL897195(16S)	this paper
L. kuhlii	NK 0039	Indonesia: Java: Java Tengah: Cilacap	-7.623	108.943	SAMN24058431	Pending(12S) OL897193(16S)	this paper
L. kuhlii	ENS 19954	Indonesia: Java: Java Tengah: Banyumas	-7.312	109.236	SAMN24058406	Pending(12S) OL897191(16S)	this paper
L. kuhlii	ENS 19941	Indonesia: Java: Java Tengah: Banyumas	-7.312	109.236	SAMN24058405	Pending(12S) OL897190(16S)	this paper
L. kuhlii	ENS 19968	Indonesia: Java: Java Tengah: Banyumas	-7.312	109.236	SAMN24058407	Pending(12S) OL897192(16S)	this paper
L. kuhlii	ENS 19860	Indonesia: Java: Java Timur: Magetan	-7.677	111.204	SAMN24058402	Pending(12S) OL897188(16S)	this paper
L. sisikdagu	ENS 18805	Indonesia: Sumatra: Aceh: Aceh Besar	5.264	95.540	SAMN24058384	Pending(12S) OL897210(16S)	this paper
L. kuhlii	ENS 15908	Indonesia: Java: Java Barat: Kabupaten Cianjur	-6.742	107.006	SAMN24058344	Pending(12S) OL897207(16S)	this paper
L. sisikdagu	ENS 14226	Indonesia: Sumatra: Lampung: Kabupaten Tanggamus	-5.282	104.557	SAMN24058323	Pending(12S) OL897211(16S)	this paper
L. kuhlii	ENS 20449	Indonesia: Borneo: Kalimantan Timur: Berau Berat	1.655	117.085	SAMN24058419	Pending(12S) OL897232(16S)	this paper
L. sisikdagu	ENS 19059	Indonesia: Sumatra: Aceh	4.474	96.541	SAMN24058388	Pending(12S) OL897182(16S)	this paper
L. sisikdagu	ENS 19058	Indonesia: Sumatra: Aceh	4.474	96.541	SAMN24058387	Pending(12S) OL897181(16S)	this paper
L. sisikdagu	ENS 20115	Indonesia: Sumatra: Aceh: Pidie Jaya	4.653	96.092	SAMN24058418	Pending(12S) OL897184(16S)	this paper
L. sisikdagu	ENS 15513	Indonesia: Sumatra: Sumatra Utara: Deli Serdang	3.337	98.584	SAMN24058338	Pending(12S) OL897218(16S)	this paper

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. sisikdagu	ENS 20061	Indonesia: Sumatra: Aceh: Gayo Lues	3.983	96.864	SAMN24058414	Pending(12S) OL897178(16S)	this paper
L. sisikdagu	ENS 18077	Indonesia: Sumatra: Bengkulu	-3.924	103.239	SAMN24058372	Pending(12S) OL897185(16S)	this paper
L. sisikdagu	ENS 19008	Indonesia: Sumatra: Aceh: Aceh Besar	5.259	95.543	SAMN24058385	Pending(12S) OL897179(16S)	this paper
L. sisikdagu	ENS 18314	Indonesia: Sumatra: Aceh: Aceh Besar	5.454	95.688	SAMN24058376	Pending(12S) OL897180(16S)	this paper
L. kuhlii	NK 0284	Indonesia: Java: Java Tengah: Wonosobo	-7.346	109.918	SAMN24058437	N/A	this paper
L. kuhlii	NK 0287	Indonesia: Java: Java Tengah: Wonosobo	-7.348	109.918	SAMN24058438	N/A	this paper
L. sisikdagu	ENS 20113	Indonesia: Sumatra: Aceh: Pidie Jaya	4.653	96.092	SAMN24058417	N/A	this paper
L. sisikdagu	ENS 19057	Indonesia: Sumatra: Aceh	4.474	96.541	SAMN24058386	N/A	this paper
L. sisikdagu	ENS 14425	Indonesia: Sumatra: Sumatra Barat	-2.041	101.315	SAMN24058324	N/A	this paper
L. sisikdagu	ENS 18733	Indonesia: Sumatra: Bengkulu: Kapahiang City	-3.659	102.558	SAMN24058382	N/A	this paper
L. kuhlii	ENS 15097	Indonesia: Java: Banten: Pandeglang	-6.336	105.970	SAMN24058336	N/A	this paper
L. sisikdagu	ENS 14526	Indonesia: Sumatra: Lampung: Kabupaten Tanggamus	-5.315	104.530	SAMN24058327	N/A	this paper
L. sisikdagu	ENS 20046	Indonesia: Sumatra: Aceh: Gayo Lues	3.982	96.864	SAMN24058411	N/A	this paper
L. kuhlii	ENS 15051	Indonesia: Java: Banten: Pandeglang	-6.331	105.969	SAMN24058332	N/A	this paper

Species Name	Museum/Field Number	Location	Lat	Long	DDRad-seq	12s and 16S	Citation
L. kuhlii	MZB 22298 ENS 15907	Indonesia: Java: Java Barat: Kabupaten Cianjur	-6.742	107.006	SAMN24058343	N/A	this paper
L. kuhlii	ENS 15914	Indonesia: Java: Java Barat: Kabupaten Cianjur	-6.742	107.006	SAMN24058350	N/A	this paper

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