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Bioacoustics Reveal Hidden Diversity in Frogs: Two New Species of the Genus *Limnonectes* from Myanmar (Amphibia, Anura, Dicroglossidae)

Gunther Köhler [1], Britta Zwitzers [1], Ni Lar Than [2], Deepak Kumar Gupta [3], Axel Janke [3,4], Steffen U. Pauls [1,3,5] and Panupong Thammachoti [6,*]

1 Senckenberg Forschungsinstitut und Naturmuseum, Senckenberganlage 25, 60325 Frankfurt a.M., Germany; gkoehler@senckenberg.de (G.K.); b.zwitzers@aol.com (B.Z.); steffen.pauls@senckenberg.de (S.U.P.)
2 Zoology Department, East Yangon University, Thanlyin 11291, Yangon, Myanmar; nilarthan65@gmail.com
3 LOEWE Centre for Translational Biodiversity Genomics (LOEWE-TBG), Senckenberganlage 25, 60325 Frankfurt a.M., Germany; deepak.gupta@senckenberg.de (D.K.G.); axel.janke@senckenberg.de (A.J.)
4 Senckenberg Biodiversity and Climate Research Centre, Georg-Voigt-Straße 14-16, 60325 Frankfurt a.M., Germany
5 Institute of Insect Biotechnology, Justus-Liebig-University Gießen, Heinrich-Buff-Ring 26-32, 35392 Gießen, Germany
6 Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
* Correspondence: panupong.th@chula.ac.th

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Abstract: Striking geographic variation in male advertisement calls was observed in frogs formerly referred to as *Limnonectes doriae* and *L. limborgi*, respectively. Subsequent analyses of mtDNA and external morphological data brought supporting evidence for the recognition of these populations as distinct species. We describe two new frog species of the genus *Limnonectes* (i.e., *L. bagoensis* sp. nov. and *L. bagoyoma* sp. nov.) from Myanmar. *Limnonectes bagoensis* sp. nov. is closely related to *L. doriae* whereas *L. bagoyoma* sp. nov. is closely related to *L. limborgi*. Results of this integrative study provide evidence for the presence of additional undescribed species in these species complexes but due to the lack of bioacoustical data, we consider these additional diverging populations as candidate species that need further study to resolve their respective taxonomic status. Both new species are distributed in Lower Myanmar. *Limnonectes doriae* is restricted to southern Myanmar along the Malayan Peninsula whereas *L. limborgi* is known to occur in eastern Myanmar and northwestern Thailand. The remaining populations formerly referred to as either *L. doriae* or *L. limborgi* are considered representatives of various candidate species that await further study. We further provide a de novo draft genome of the respective holotypes of *L. bagoensis* sp. nov. and *L. bagoyoma* sp. nov. based on short-read sequencing technology to 25-fold coverage.

Keywords: bioacoustics; cryptic species diversity; Dicroglossidae; genome; *Limnonectes bagoensis* sp. nov.; *Limnonectes bagoyoma* sp. nov.; Myanmar; new species; Thailand

1. Introduction

The anuran family Dicroglossidae has its center of distribution and greatest species diversity in South, Southeast, and East Asia as well as on the islands of the Sunda Shelf and the Philippines but is also known from northwestern and sub-Saharan Africa across the southern Arabian Peninsula to Pakistan [1]. The great number of new species described every year (e.g., [2–4]) shows that this group of frogs is understudied. Obviously, much more field and lab work is needed to evaluate the actual diversity and better understand

their phylogenetic relationships. Additionally, many of the supposedly widespread species actually consist of several species. This further drives the discovery of new species with intensified study (e.g., [5–8]).

During fieldwork in Myanmar, we came across variations in the male advertisement call of two frog species that belong to the genus *Limnonectes*, *L. doriae* (Boulenger, 1887) and *L. limborgi* (Sclater, 1892), respectively. In fact, the calls were so different that we immediately suspected multiple species and initiated a taxonomic study of these two species complexes to evaluate if the bioacoustically distinct populations were also differentiated in morphology and molecular genetics and warrant describing them as distinct species.

The species currently recognized as *L. limborgi* was originally described by Sclater (1892) as *Rana limborgi*. The holotype, however, is lost and a neotype (specimen MSNG 29422) collected by Leonard Fea at "Karin Bia-po", Myanmar, was designated [9]. In the descriptions of his travel activities in Myanmar, Fea [10] reported that some of his zoological collections were made at "Leitò, villaggio di Carin Chebà o Bia-Po, posto a 900 metri sul livello del mare" (=Leitò, village of Carin Chebà or Bia-Po, located at 900 m above sea level). Today this village is called Leiktho and is located at 19.2212°, 96.5818°, 865–885 m above sea level. Other authors published erroneous GPS coordinates for Lea's "Bia-Po" such as "19°03′ N/96°30′ E" (=19.05°, 96.50°) [9] or "19°15′ N/97°30′ E" (=19.25°, 97.50°) [11]. The names Carin or Karin refer to the Independent State of Carin that included "Leitò" at the time of Lea's travels in Myanmar. Today, this village is in Kayin State.

Dubois [12] established a new genus, *Taylorana*, to accommodate the species of *Limnonectes* with direct development, comprising *L. hascheanus* and *L. limborgi*. However, phylogenetic analyses show (e.g., [13,14]) that these two species are nested within the remaining species currently assigned to *Limnonectes*. Thus, the placement of these two species in *Taylorana* was never widely accepted, though some authors recognized *Taylorana* as a subgenus within *Limnonectes* (e.g., [9,15]). Smith [16] considered *L. hascheanus* and *L. limborgi* to be synonyms until *L. limborgi* was resurrected from synonymy with *L. hascheanus* by Dubois [12], and Inger and Stuart [9] demonstrated that these two nominal species differ in body size, degree of toe webbing, development of odontoids in adult males as well as in molecular genetics.

In 1887, Boulenger [16] introduced his new species *Rana doriae* based on material he had obtained from Leonardo Fea. The geographic origin of the type series is given as "Thagatà Juwa; Kaw-ka-riet" (now called Kawkareik, about 66 km east of the city of Mawlamyine, northern Tanintharyi, Myanmar; see also [1]). The GPS coordinates for this locality were given as 16°33′ N, 98°14′ E [11]. Ohler and Dubois [17] restricted the geographic distribution of *L. doriae* to the Tenasserim region in Myanmar and adjacent Thailand on the Malayan Peninsula. However, Inger and Stuart [9] identified a specimen from Bago Yoma, Bago Region, Myanmar, as *L. doriae*.

Smith [18] associated *Rana doriae* with a group of species that all tend to develop a swelling or flap-like structure in the occipital region on top of the head as well as the presence of well-developed odontoids on the lower jaw on either side of the symphysis. Later, the species was transferred to the genus *Limnonectes* [12]. Ohler and Dubois [17] placed *Limnonectes doriae* in the subgenus *Elachyglossa* along with *L. dabanus*, *L. gyldenstolpei* (=the type species of this subgenus), *L. kohchangae*, *L. macrognathus*, *L. maw-phlangensis*, *L. plicatellus*, and *L. toumano*. Lambertz et al. [19] restricted the subgenus *Elachyglossa* to the four caruncle-bearing species of *Limnonectes* that were recognized at the time (i.e., *L. dabanus*, *L. gyldenstolpei*, *L. macrognathus*, and *L. plicatellus*). However, the phylogeny published by Phimmachak et al. [20] indicated *L. kohchangae*, *L. macrognathus*, and *L. plicatellus* to be more closely related to the non-caruncle-bearing species *L. doriae*, *L. hascheanus*, and *L. limborgi* than to the other caruncle-bearing species (i.e., *L. dabanus*, *L. gyldenstolpei*, *L. lauhachindai*, *L. savan*). Most recently, Yodthong et al. [21] described a new species related to *L. doriae*. Obviously, additional phylogenetic studies are needed including also nuclear DNA loci in order to resolve the relationships of the species of *Limnonectes*.

2. Materials and Methods

The methods and format of the presentation follow previous works coauthored by the senior author [2,5,22]. Specimens examined for this study were collected by GK, NLT, and PT (see Appendix A for specimens examined). Specimens labeled with GK field numbers were deposited in the collections of Senckenberg Forschungsmuseum Frankfurt (SMF) or at East Yangon University (EYU), Thanlyin, Myanmar, and those with PT field numbers were deposited in the collection of the Chulalongkorn University Museum of Natural History (CUMZ), Bangkok.

Prior to the preservation of collected specimens in the field, we took color photographs of each individual in life. We euthanized the frogs with a pericardial injection of T61 or MS 222. We cut tissue samples from one forelimb or from the tongue and preserved these in 98% non-denatured ethanol for DNA extraction. The tissue samples were deposited in the collection of SMF and CUMZ. Specimens were then preserved by injecting a solution of 5 mL absolute (i.e., 36%) formalin in 1 L of 96% ethanol into the body cavity, and stored in 70% ethanol. Coordinates and elevation were recorded using Garmin GPS receivers with built-in altimeters. All coordinates were recorded in decimal degrees, WGS 1984 datum. Capitalized colors and color codes (in parentheses) followed [23].

In evaluating species' boundaries among populations, we followed the evolutionary species concept [24,25]. As lines of evidence for species delimitation, we applied a phenotypic criterion (external morphology), the genetic distinctness of a mitochondrial genetic marker as well as a criterion for reproductive isolation (bioacoustic data).

Abbreviations used are EYD (eye diameter); FL (foot length); HL (head length); HW (head width); IND (internasal diameter); IOD (interorbital diameter); LAL (lower arm length; measured from elbow to base of palmar tubercle); NED (nostril–eye distance); HNL (hand length), measured from base of palmar tubercle to tip of third finger); SHL (shank length); SL (snout length); SVL (snout–vent length); TED (tympanum–eye distance); THL (thigh length); TYD (longitudinal tympanum diameter); UEW (upper eyelid width). Webbing formulae follow [26]. Terminology of snout shape follows [27]. Morphological data for *Limnonectes alpinus*, *L. liui*, and *L. medogensis* were taken from [15].

We recorded anuran vocalizations using a digital audio recorder (Olympus LS-12) with a Sennheiser ME 66 shotgun microphone capsule and a Sennheiser K6 powering module. The microphone was positioned between 0.5 and 1.5 m from the calling frog. Aside from the GPS coordinates and elevation above sea level of the locality we also noted ambient air temperature and determined the SVL of the recorded individual. Files were recorded as uncompressed 24-bit WAV files at a sampling frequency of 96 kHz. Audio files were deposited in the Fonoteca Zoológica, Museo Nacional de Ciencias Naturales, Madrid, Spain.

The spectral and temporal parameters were analyzed and the power spectra were calculated in RAVEN PRO 1.4. (Bioacoustics Research Program 2011). Spectrograms were obtained using the Blackman window function at 3db Filter Bandwidth of 158 Hz; grid spacing of 21.5 Hz; overlap 90%. Frequency information was generated through Fast Fourier Transformation (FFT, width 2048 samples). Temporal measurements of calls such as repetition rates, duration of notes, and the number of pulses, were measured on the waveforms. Terminology in call descriptions follows [28]. The map was created using ArcMap 10.4.

Marker-based analysis: We extracted DNA following the protocol of [29]. To eliminate potential PCR-inhibiting contaminants, the tissue samples were incubated for 14 h at 4 °C in 200 µL low PBS buffer (20 µL PBS in 180 µL of water) before overnight digestion with the vertebrate lysis buffer at 56 °C. After extraction, DNA was eluted in 50 µL TE buffer. Fragments of the mitochondrial 16S rRNA (16S) were amplified in an Eppendorf Mastercycler® Pro using the following protocol: initial denaturation for 2 min at 94 °C; followed by 40 cycles with denaturation for 35 s at 94 °C, hybridization for 35 s at 48.5 °C, and elongation for 60 s at 72 °C; final elongation for 10 min at 72 °C. The reaction mix for each sample contained 1 µL DNA template, 14 µL water, 2.5 µL PCR-buffer, 1 µL 25 mM

$MgCl_2$, 4 µL 2.5 mM dNTPs (Invitrogen), 0.5 µL (containing 2.5 units) Taq Polymerase (PeqLab), and 1 µL of the primer (16S: forward: L2510, 5′–CGCCTGTTTATCAAAAACAT–3′; reverse: H3056, 5′–CCGGTCTGAACTCAGATCACGT–3′; from Eurofins MWG Operon). DNA extraction, PCR, and sequencing were performed at SMF for samples from Myanmar and at CUMZ for samples from Thailand. We generated 43 new sequences for this study (see Appendix B). Additionally, we downloaded relevant 16S sequences from GenBank (Appendix B). Our dataset contains topotypic specimens of *Limnonectes limborgi* and near-topotypic specimens of *L. doriae*.

We aligned the sequences with MUSCLE [30] using the default settings in Geneious 6.1.2. [31]. For software applications, sequence data formatting was converted using the online server Alter [32]. The best substitution model for each gene was identified using PartitionFinder2 [33], with linked branch lengths via PhyML 3.0 [34]. Model selection used the corrected (for finite sample size) Akaike Information Criterion (AICc) [35]. *Fejervarya limnocharis* (GenBank number AF206466) was used as an outgroup [14].

Bayesian Inference analyses (BI) used MrBayes 3.2 [36] with five runs with eight chains. The first 25% of trees were discarded as burn-in. MCMC runs used an initial set of 1,000,000 generations with sampling every 500 generations and adding 500,000 generations until chains reached convergence. Convergence was considered achieved when the standard deviation of split frequencies was 0.015 or less. Additionally, convergence was diagnosed by PSRF (Potential Scale Reduction Factor), which should approach 1.0 as runs converge [37]. We used the IQTree webserver [38] to run a Maximum Likelihood (ML) analysis using 10,000 ultrafast Bootstrap approximation (UFBoot) replicates with 10,000 maximum iterations and minimum correlation coefficient of 0.99 [39], plus 10,000 replicates of Shimodaira–Hasegawa approximate likelihood ratio (SH-aLRT), which proved to be accurate with a high statistical power [34]. We used FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/, accessed on 2 April 2021) for viewing the trees. We estimated evolutionary genetic divergence, computing uncorrected pairwise distances with MEGA 7.0.26 [40] to assess the degree of intra- and interspecific differences, using a Bootstrap estimation method of 10,000 replications. We built a species tree using BEAST 2.4.7 [41,42] with 1,000,000 generations for the MCMC model. The resulting tree was visualized using DensiTree 2.2.6 [43].

Whole-genome sequencing and assembly: Genomic DNA was extracted from muscle tissue of the respective holotypes (SMF 104090 and SMF 106034) of the two new species of *Limnonectes* described herein according to standard phenol/chloroform procedures [44]. The resulting DNA was then resuspended in TE buffer (10 mM Tris-Cl, 0.1 mM EDTA) and stored at −20 °C. Quality checks for high molecular weight DNA were performed by agarose gel electrophoresis [44]. The DNA samples were shipped on dry ice to Novogene (UK) for short-read Illumina genome sequencing. One genomic library for each of the holotypes (insert size: 350 bp) was prepared and 150 bp paired-end reads were sequenced on an Illumina NovaSeq 6000 platform (San Diego, CA, USA). A long-read genomic sequencing was performed for the holotype SMF 106034 using PacBio Sequel sequencing platform.

A *k*-mer profile analysis was performed from the raw reads with the help of Jellyfish 2.3.0 software [45] and GenomeScope webserver [46]. The quality checks of the raw reads were performed by trimming the low-quality regions and adapter sequences and filtered for possible contamination using Trimmomatic 0.39 [47] and Kraken 2.0.9 [48], respectively. Nuclear genome assembly of the holotype SMF 106034 was conducted in the SPAdes assembler [49]. Scaffolds smaller than 500 in length were discarded. To assemble the nuclear genome of the holotype SMF 104090, the short reads were mapped over the genome of SMF 106034 with the help of Bowtie2 software [50]. The resulting SAM file was then converted to a sorted bam file by using Samtools [51]. The consensus sequence was then generated with the help of Samtools and BCFtools [52] software. Scaffolds smaller than 500 in length were discarded.

The mitochondrial genome of SMF 104090 was assembled from the genomic data separately using NOVOplasty 4.2 [53]. For the holotype, SMF 106034, mitochondrial genome of the holotype SMF 104090 was taken as reference and Pacbio reads which had homology to the reference were fetched using NCBI Blast 2.11 [54]. These reads were then used in Geneious Prime software 2020.2.3 (www.geneious.com) to generate the consensus sequence. The consensus sequence was then polished by Illumina short reads using Pilon v1.23 software [55]. Annotation of the mitochondrial genome was performed by MITOS2 webserver [56] and visualization pictures were generated using Geneious Prime software 2020.2.3.

3. Results and Conclusions

The alignment of the 16S sequences used in this study had a length of 583 bp. General Time Reversible using a discrete Gamma distribution with five rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (GTR + I + G) was determined to be the best fitting model of sequence evolution. The trees generated by BI, ML, and BEAST analyses agree well at most well-supported nodes, but show differences in branch arrangement at weakly supported nodes (Figures 1 and 2).

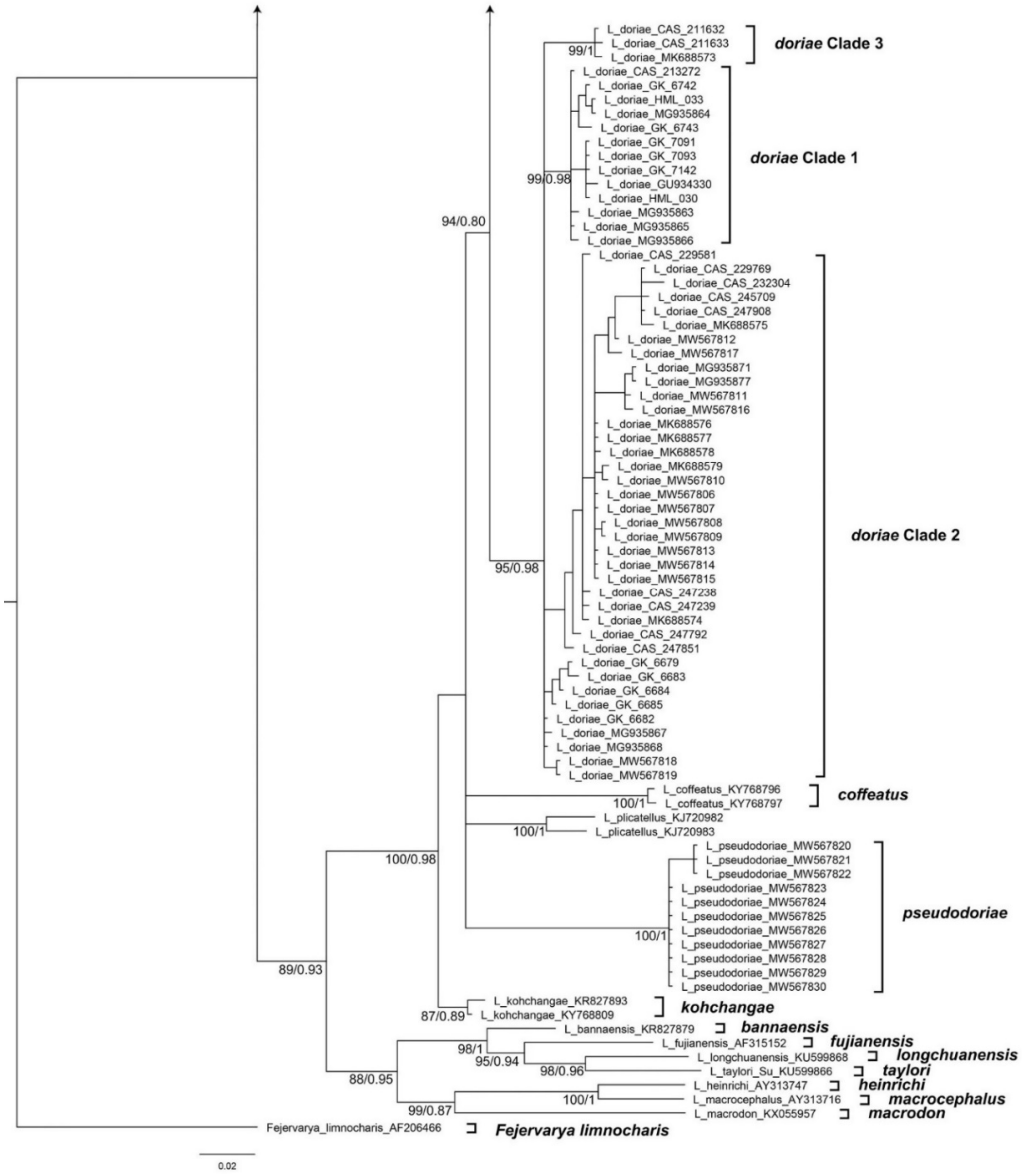

Figure 1. *Cont.*

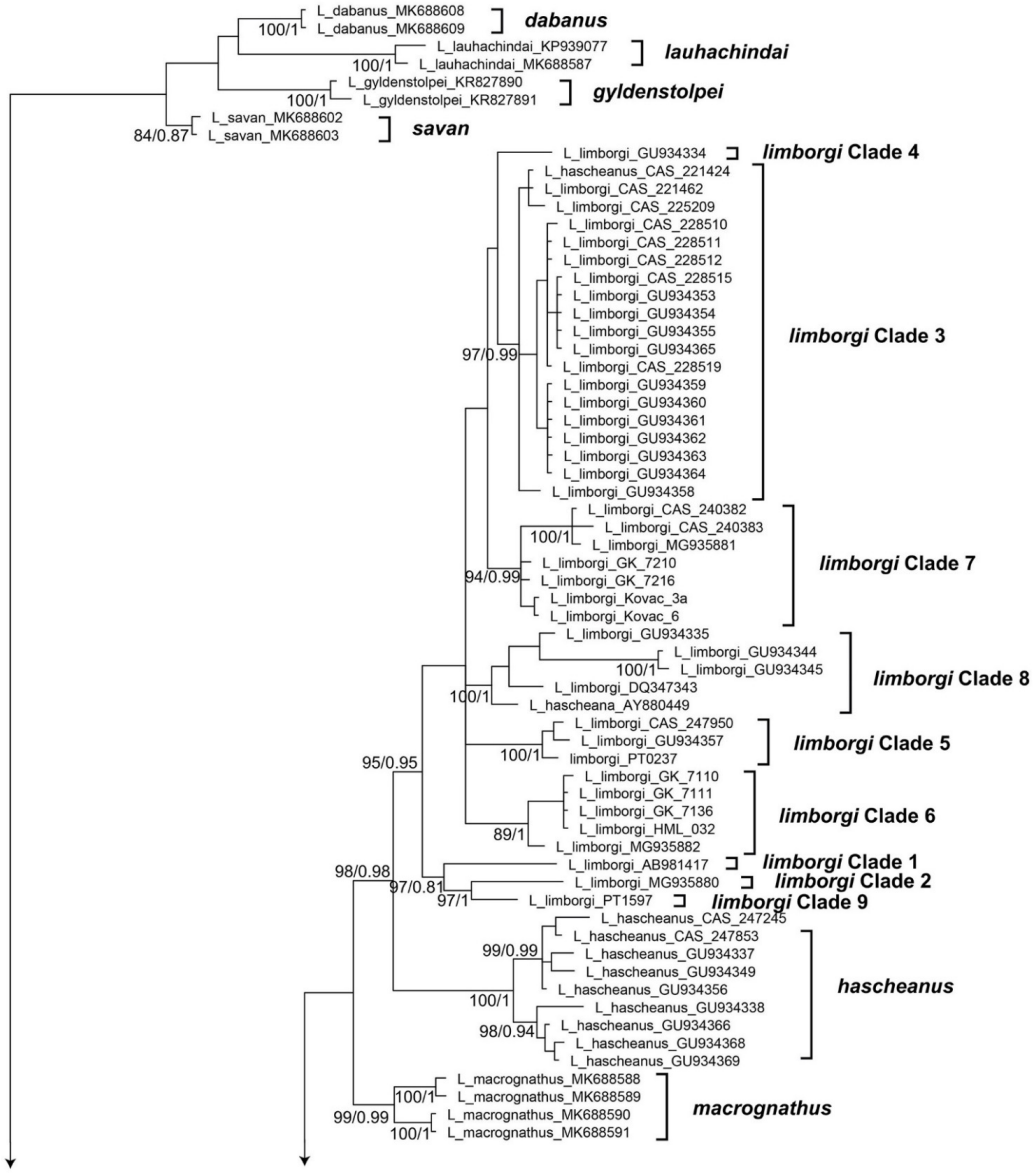

Figure 1. Phylogenetic relationships among frogs of the genus *Limnonectes* from a BI analysis inferred from the mtDNA marker 16S. The numbers at nodes are bootstrap values (left) and Bayesian posterior probabilities (right), but only for nodes with bootstrap values higher than 75. A scale bar of genetic distance is indicated. The tree is rooted using *Fejervarya limnocharis*.

Limnonectes doriae species complex. Among the specimens formerly referred to as *Limnonectes doriae*, three major clades are recognizable (Figure 1). Clade 1 contains specimens from lower Myanmar in Bago and Yangon Regions (Figure 3). Clade 2 includes samples from southern Myanmar including the Malayan Peninsula and adjacent regions in extreme western Thailand; only in the ML analysis, these specimens formed a weakly supported clade, whereas, in the Bayes analysis, these are recovered as a polytomy. Finally, Clade 3 contains specimens from western Myanmar in Rakhine state. The genetic distances among these clades vary from 1.9% to 2.4% (Table 1). We consider these three clades as candidate species, that should be assessed by including additional data types. Clade 3 includes specimens from near the type locality of *L. doriae* and is therefore considered to represent the "true" *L. doriae*.

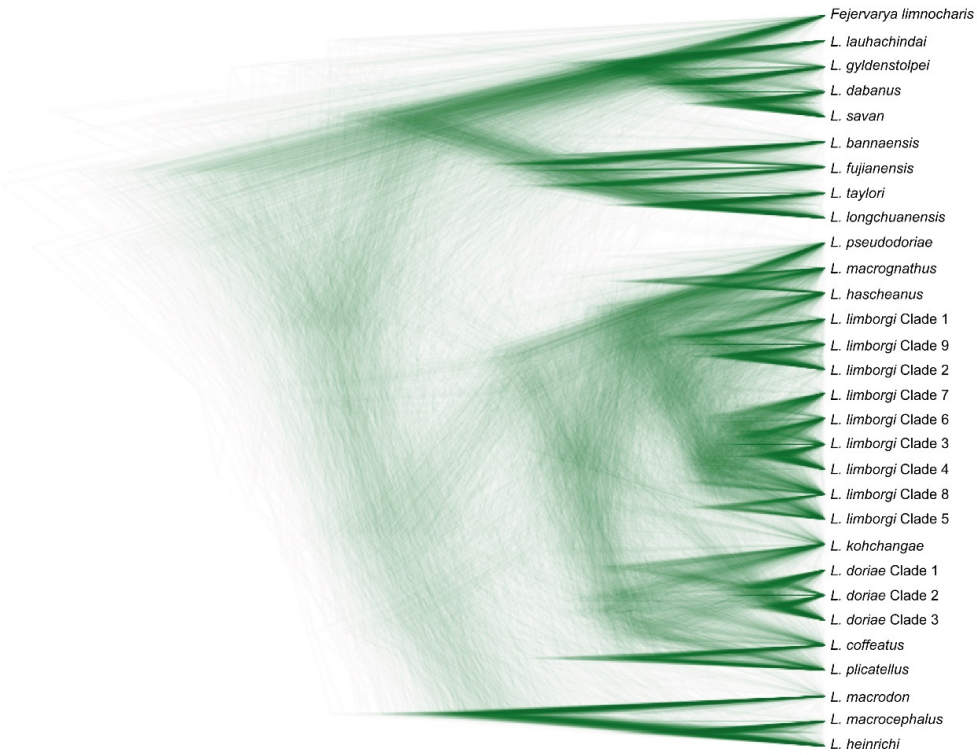

Figure 2. Species tree indicating phylogenetic relationships among frogs of the genus *Limnonectes* from a BEAST analysis showing density of trees proportional to frequency of occurrence drawn in DensiTree.

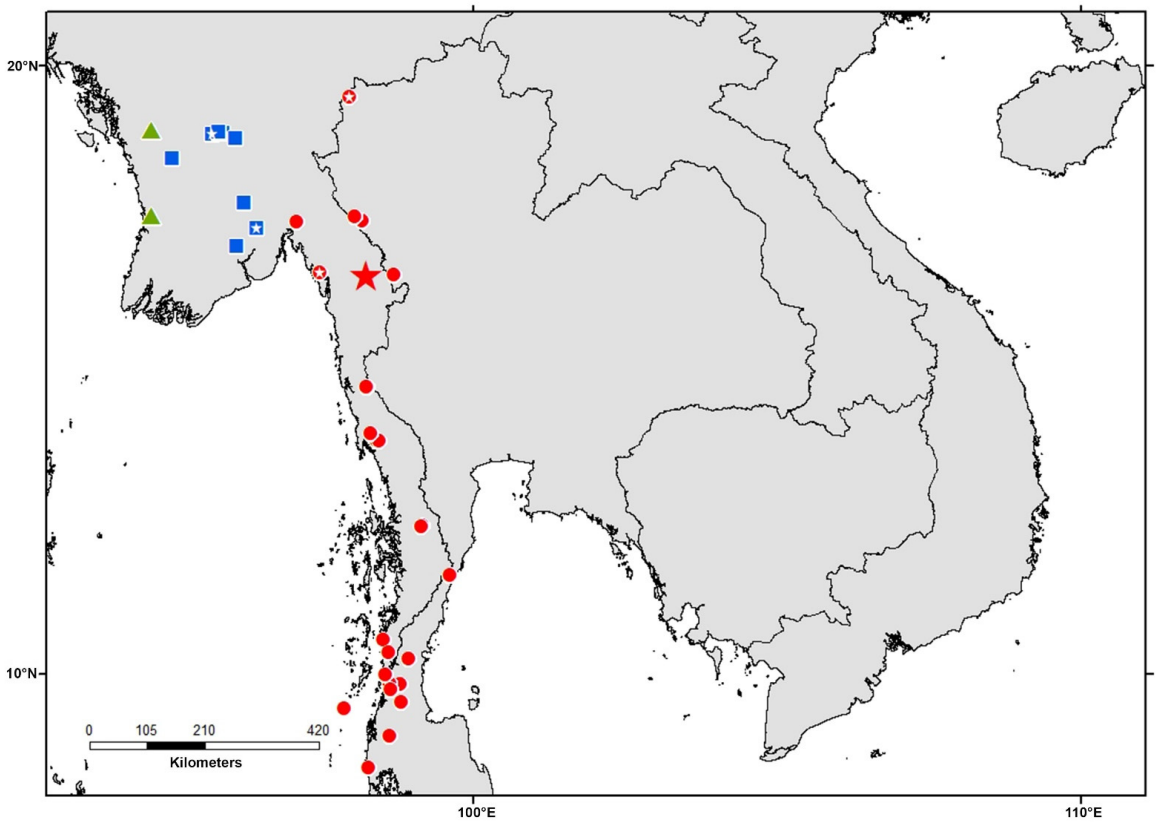

Figure 3. Map showing collecting localities of the *Limnonectes* species related to *L. doriae* included in this study Each symbol can represent one or more adjacent localities. Blue squares = Clade 1; red circles = Clade 2; green triangles = Clade 3; red star = type locality of *L. doriae*. Small white stars within symbols indicate localities from which bioacoustics data have been used in this study.

Table 1. Mean genetic distances (in %) of Clades 1–3 (see Figure 1) of frogs referred to as *Limnonectes doriae* based on the 16S dataset. See text for details.

	Clade_3	Clade_1
Clade_3		
Clade_1	2.4%	
Clade_2	2.5%	1.9%

Bioacoustical data are available for our Clades 1 and 2 (see also Table 2). The male advertisement calls of these two clades are strikingly different (Figure 4; Table 2): The call of SMF 104084 from Clade 2 from the region of the species' type locality (Figure 4A) consists of a single series of 15–26 notes, each note varying from 51 to 103 ms (mean 65 ms) in duration with 11.6–12.1, mean 11.8, notes per second, their amplitude rising during the first 3–8 notes, then remaining about the same level. There is no offset single note anterior to the series of notes. The call has a duration of 1.25–2.24 s and a dominant frequency of 689–990 Hz, a mean of 947 Hz. The analyzed call of a Clade 2 male (ZMKU 1552) from a locality in Mae Hong Son Province, Thailand, about 320 airline km NNE of the SMF 104084 locality (see Figure 3), is similar in structure (i.e., a single series of notes without an off-set single note anterior to this series), dominant frequency (840–1055 Hz, mean 902 Hz), note duration (60–72 ms, mean 68 ms), and number of notes per second (11.7). It differs in the length of the call (6.75–7.71 s) and thus in the number of notes per call (79–90, mean 83.7). Whether this difference in call duration is of individual or geographical nature can only be clarified once the calls of additional individuals and localities can be included in the analyses.

Table 2. Selected bioacoustic parameters of the species related to *Limnonectes doriae.* Range is followed by mean value and standard deviation in parentheses. Dom. Freq. = dominant frequency; Freg. = frequency.

	Taxon ID	Call Duration (sec)	Dom. Freq (Hz)	Gap Duration (sec)	Notes Per Call	Notes/s	Freq 5% (Hz)	Freq 95% (Hz)
SMF 104084_0170 (n = 12 calls)	*L. doriae*, Clade 3, near Mawlamyine	1.25–2.24 (1.74 ± 0.32)	775–1033 (947 ± 80.6)	2.29–8.43 (4.39 ± 1.94)	15–26 (20.6 ± 3.6)	11.6–12.1 (11.8 ± 0.15)	302–517 (398 ± 68)	1292–1507 (1385 ± 52)
ZMKU 1552 (n = 3 calls)	*L. doriae*, Clade 3, Thailand, Mae Hong Son Province	6.75–7.71 (7.16 ± 0.40)	840–1055 (902 ± 51.7)	32.2	79–90 (83.7 ± 4.64)	11.7–11.7 (11.7 ± 0.02)	345–431 (367 ± 19)	1335–1486 (1414 ± 53)
SMF 104090_0185 (n = 10 calls)	*L.* cf. *doriae*, Clade 1, Bago	2.09–3.27 (2.72 ± 0.32)	861–991 (945 ± 30.6)	5.11–10.82 (7.42 ± 2.44)	5–9 (7.1 ± 1.0)	4.2–4.6 (4.4 ± 0.14)	431–495 (458 ± 19)	1271–1335 (1302 ± 19)
SMF 106039_418 (n = 5 calls)	*L.* cf. *doriae*, Clade 1, Bago Yoma	2.62–3.32 (2.99 ± 0.24)	732–818 (772 ± 21.2)	49.25–72.67 (59.64 ± 9.74)	7–8 (7.2 ± 0.4)	3.6–4.1 (3.9 ± 0.19)	345–388 (367 ± 22)	1163–1206 (1201 ± 13)

The call of Clade 1 males (Figure 4C,D) consists of two portions that are separated by a break of 573–1043 ms (mean 792 ms). The first portion of the call is a single unpulsed sound emission (note duration 225–291 ms) and with a slightly weaker amplitude than the second portion which consists of 4–9 notes. Depending on the number of notes, the second portion of the call has a duration of 1202 (4 notes) to 1954 ms (9 notes), mostly around 1600 ms (7 notes); each note varying from 155 to 239 ms in duration with 4.2–4.6, mean 4.4, notes per second, their amplitude rising during the first note, then remaining about the same level. The dominant frequency is at 861–990 Hz (mean 945 Hz) for both call portions. We analyzed the advertisement calls of Clade 1 males (Figure 4C,D) from two localities that are about 190 air-line km apart from another (see Figure 3), and the calls

from the different localities agree very well in all examined characters (see Table 2). The mean genetic distance (16S) between Clade 1 and 2 is 1.9% (Table 1).

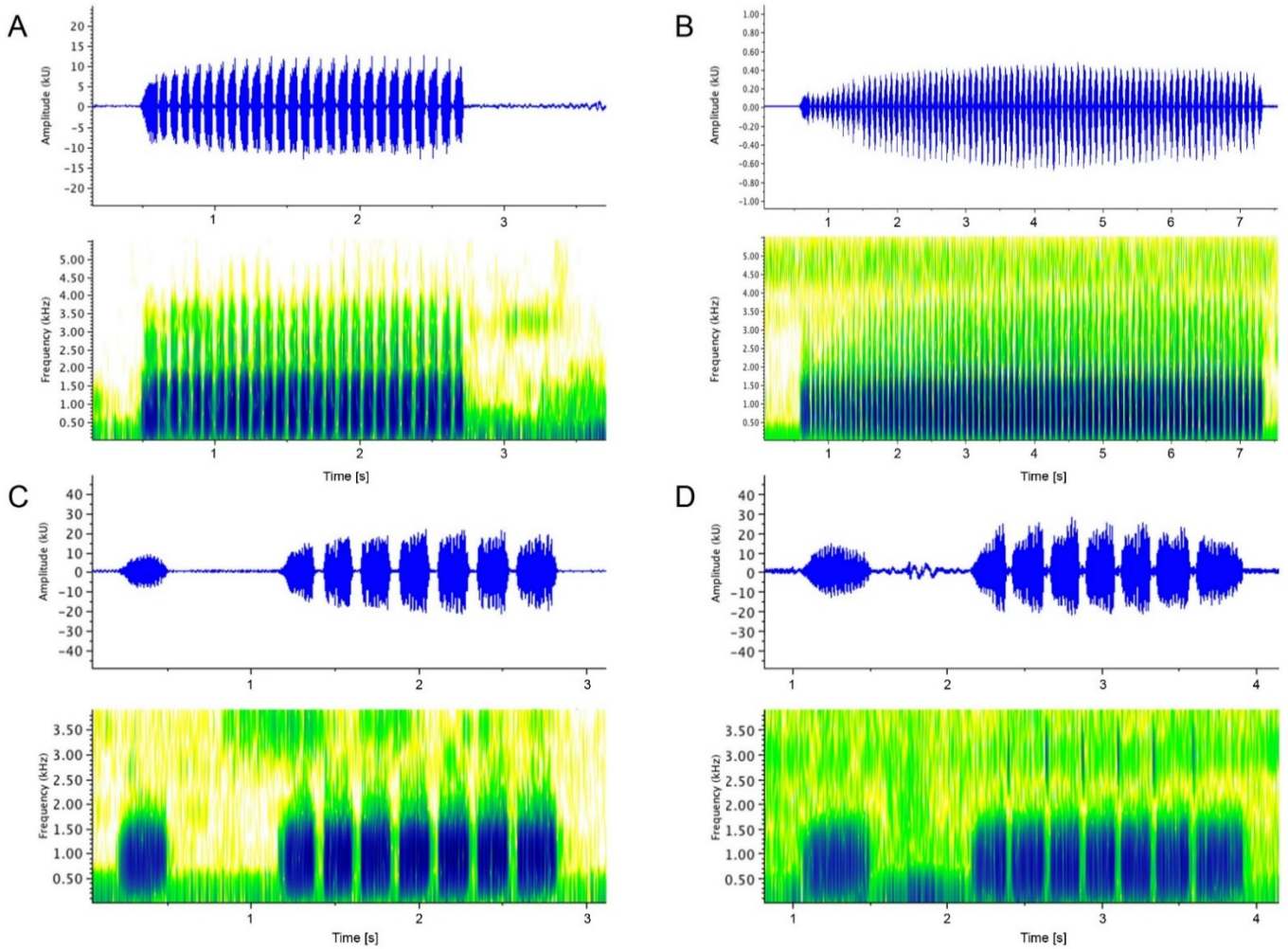

Figure 4. Advertisement calls of male *Limnonectes*. (**A**) *L. doriae*, SMF 104084 (Mon State, Myanmar); (**B**) *L. doriae*, ZMKU 1552 (Mae Hong Son Province, Thailand); (**C**) *L. bagoensis* n. sp., SMF 104090 (Bago, Bago Region, Myanmar); (**D**) *L. bagoensis* n. sp., SMF 106039 (Bagoyoma, Bago Region, Myanmar). See text for details and Figure 4 for localities plotted on map.

In external morphology, specimens of these three clades are very similar but show differences in some morphometric values (Figure 5) as well as in the amount of toe webbing and in some details of coloration (see Table 3 and respective diagnosis sections below). Additionally, there is sexual dimorphism evident in several morphometric characters such as SVL, IOD, TYD, and HL. In particular, individuals of Clades 1 and 2 differ in the pattern on the posterior surface of the thigh (distinct and contrasting in Clade 1 versus weak and indistinct in Clade 2), development of a dermal fringe along the outer edge of Toe 5 (absent or weak in Clade 1 versus distinct in Clade 2), and the gular coloration in adult males (pale in Clade 1 versus dark gray in Clade 2). However, since our sample sizes are small for characters of external morphology, these putative differences between the clades need to be evaluated with more specimens in order to test their actual diagnostic value.

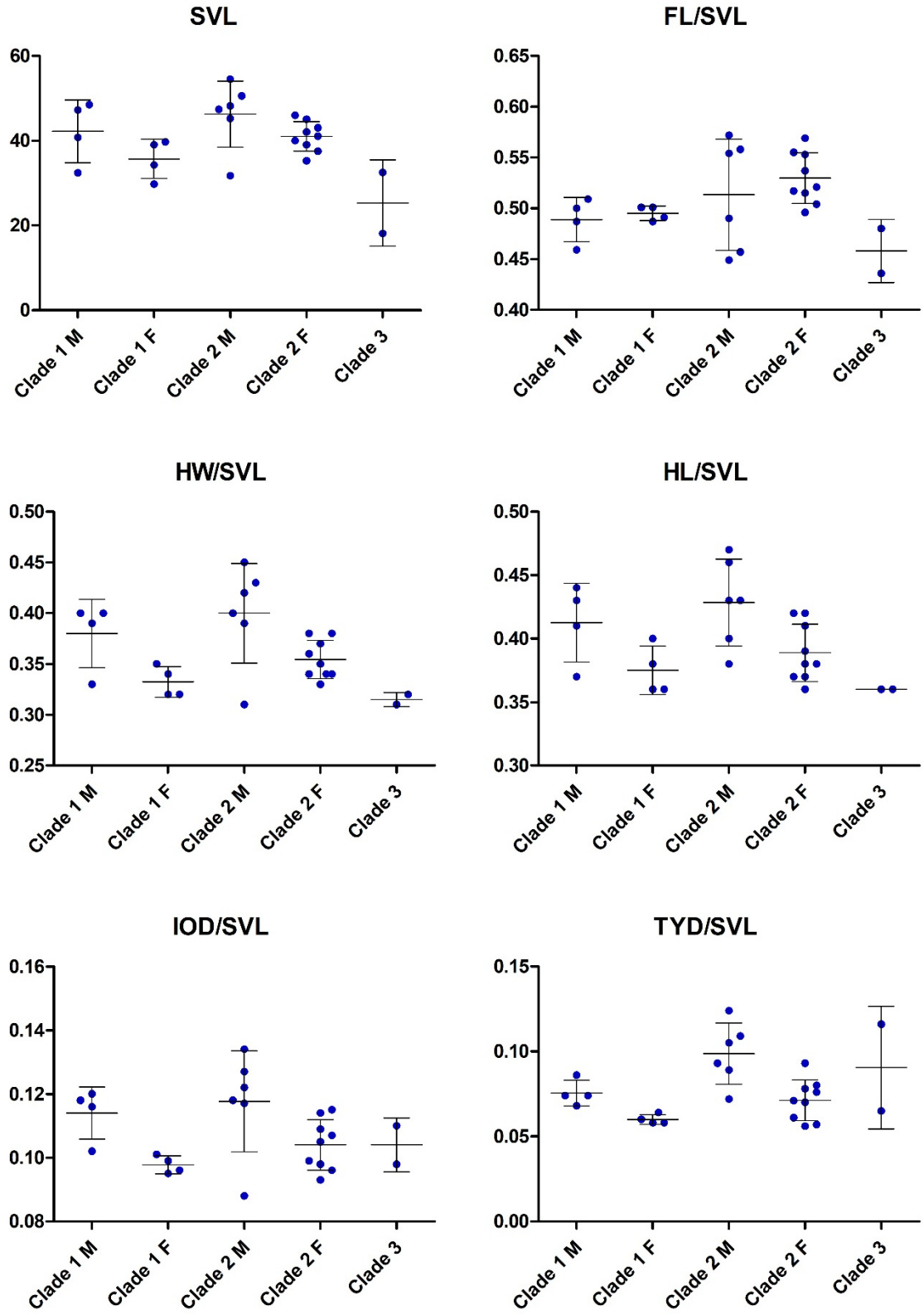

Figure 5. *Cont*.

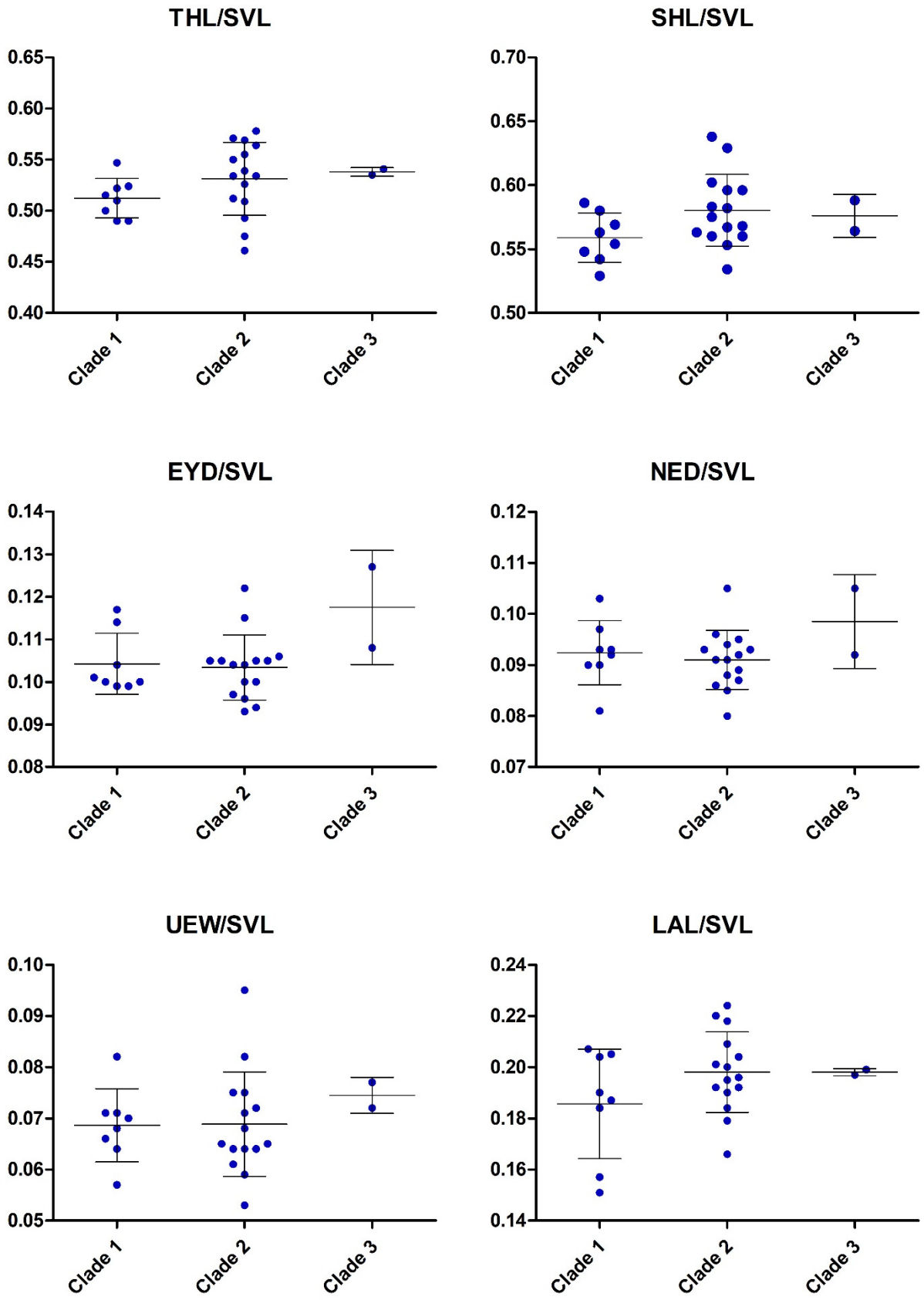

Figure 5. Scatter plots illustrating morphological variation in the clades frogs related to *Limnonectes doriae*. M = males; F = females. For definitions of Clades and abbreviations of morphological characters see text.

Table 3. Selected measurements and proportions of the two new species of *Limnonectes* described herein and the species they are most closely related to. Range is followed by mean value and standard deviation in parentheses. For abbreviations see text.

		L. bagoensis ♂**4** ♀**4**	***L. doriae*** ♂**3** ♀**3**	***L. bagoyoma*** ♂**2** ♀**2**	***L. limborgi*** ♂**1** ♀**2**
SVL	males	32.40–48.50 (42.25 ± 7.387)	47.40–54.50 (50.83 ± 3.556)	29.3–30.8 (30.05 ± 1.06)	31.20
	females	29.80–39.70 (35.73 ± 4.631)	37.50–42.10 (36.57 ± 2.335)	23.2–26.8 (25.00 ± 2.55)	26.9–27.4 (27.15 ± 0.35)
SHL/SVL	males	0.529–0.586 (0.553 ± 0.024)	0.534–0.560 (0.549 ± 0.013)	0.471–0.515 (0.493 ± 0.031)	0.526
	females	0.554–0.580 (0.566 ± 0.010)	0.560–0.568 (0.563 ± 0.004)	0.522–0.522 (0.522 ± 0.0)	0.494–0.533 (0.513 ± 0.027)
FL/SVL	males	0.459–0.509 (0.488 ± 0.021)	0.449–0.490 (0.465 ± 0.021)	0.393–0.468 (0.430 ± 0.053)	0.490
	females	0.487–0.501 (0.495 ± 0.007)	0.496–0.517 (0.505 ± 0.010)	0.470–0.487 (0.480 ± 0.009)	0.446–0.493 (0.469 ± 0.033)
HL/SVL	males	0.370–0.440 (0.412 ± 0.030)	0.430–0.470 (0.453 ± 0.020)	0.390–0.400 (0.395 ± 0.007)	0.350
	females	0.360–0.400 (0.375 ± 0.019)	0.370–0.380 (0.373 ± 0.005)	0.330–0.340 (0.335 ± 0.007)	0.310–0.370 (0.340 ± 0.042)
HW / SVL	males	0.330–0.400 (0.380 ± 0.033)	0.390–0.450 (0.420 ± 0.030)	0.880–1.030 (0.955 ± 0.106)	0.350
	females	0.320–0.350 (0.332 ± 0.015)	0.340–0.350 (0.343 ± 0.005)	0.880–0.910 (0.895 ± 0.021)	0.300–0.330 (0.315 ± 0.021)
HW/HL	males	0.900–0.940 (0.920 ± 0.023)	0.910–0.960 (0.930 ± 0.026)	0.880–1.03 (0.955 ± 0.106)	1.020
	females	0.800–0.940 (0.892 ± 0.063)	0.890–0.950 (0.916 ± 0.030)	0.880–0.910 (0.895 ± 0.021)	0.890–0.960 (0.925 ± 0.049)
IOD/SVL	males	0.102–0.120 (0.114 ± 0.008)	0.118–0.134 (0.126 ± 0.008)	0.104–0.109 (0.106 ± 0.003)	0.119
	females	0.095–0.101 (0.097 ± 0.002)	0.093–0.105 (0.099 ± 0.006)	0.101–0.108 (0.104 ± 0.004)	0.104–0.109 (0.106 ± 0.003)
TYD / SVL	males	0.680–0.860 (0.075 ± 0.007)	0.089–0.109 (0.101 ± 0.010)	0.071–0.075 (0.073 ± 0.002)	0.064
	females	0.058–0.064 (0.060 ± 0.002)	0.056–0.061 (0.058 ± 0.002)	0.073–0.075 (0.074 ± 0.001)	0.077–0.082 (0.079 ± 0.003)
EYD/SVL	males	0.099–0.114 (0.103 ± 0.007)	0.094–0.105 (0.098 ± 0.005)	0.104–0.119 (0.111 ± 0.010)	0.131
	females	0.101–0.117 (0.107 ± 0.008)	0.096–0.115 (0.105 ± 0.009)	0.091–0.127 (0.109 ± 0.025)	0.126–0.128 (0.127 ± 0.001)
NED/SVL	males	0.090–0.103 (0.095 ± 0.005)	0.093–0.105 (0.097 ± 0.006)	0.065–0.075 (0.070 ± 0.007)	0.071
	females	0.081–0.093 (0.089 ± 0.005)	0.087–0.096 (0.092 ± 0.004)	0.075–0.095 (0.085 ± 0.014)	0.078–0.080 (0.079 ± 0.001)
TYD/EYD	males	0.649–0.854 (0.734 ± 0.089)	0.910–0.960 (0.930 ± 0.026)	0.629–0.688 (0.658 ± 0.041)	0.488
	females	0.500–0.641 (0.574 ± 0.057)	0.890–0.950 (0.916 ± 0.030)	0.588–0.810 (0.699 ± 0.157)	0.600–0.647 (0.623 ± 0.033)

Thus, based on the results of the integrative analyses of mtDNA data, morphology, and bioacoustics, we recognize Clades 1 and 2 as species-level units. Clade 3 remains a candidate species awaiting further study, especially based on nuclear DNA data and bioacoustics. Based on the respective type locality, our Clade 2 is assigned to *Limnonectes doriae* whereas our Clade 1 represents an undescribed species for which no name is available. Thus, we describe the *Limnonectes* related to *L. doriae* from lower Myanmar (Bago Region and Yangon Region, our Clade 1) as a new species below.

Limnonectes limborgi species complex. Among the specimens formerly referred to as *Limnonectes limborgi*, we found nine major clades that we recognize as candidate species (Figures 1 and 2). The genetic distances among these clades vary from 1.8% to 4.9%. (Table 4). Of these clades, five (i.e., Clades 1, 2, 4, 8, 9) are represented only by sequences (mostly from GenBank) and we are unable to study these in any detail due to the lack of morphological or bioacoustical data. Therefore, these clades were not included in the integrative taxonomic analysis.

Table 4. Mean genetic distances (in %) of Clades 1–12 (see Figure 1) of frogs referred to as *Limnonectes limborgi* as well as *L. hascheanus* based on the 16S dataset. Cl = Clade; hasch. = hascheanus. See text for details.

	Cl_4	**Cl_8**	**Cl_3**	**Hasch.**	**Cl_1**	**Cl_7**	**Cl_5**	**Cl_6**	**Cl_2**
Cl_4									
Cl_8	3.2%								
Cl_3	1.8%	3.1%							
hasch.	5.5%	5.6%	5.3%						
Cl_1	4.8%	4.7%	4.7%	5.6%					
Cl_7	2.2%	3.0%	2.1%	4.7%	4.5%				
Cl_5	3.4%	3.7%	3.4%	5.7%	4.8%	3.1%			
Cl_6	3.0%	3.5%	2.8%	5.4%	4.9%	2.8%	3.7%		
Cl_2	4.3%	4.5%	4.0%	5.7%	4.2%	4.4%	4.5%	4.4%	
Cl_9	4.3%	4.4%	3.8%	4.3%	3.5%	3.5%	3.9%	3.9%	2.9%

Of the remaining four clades (i.e., 3, 5–7), we also examined voucher specimens for characters of external morphology. Clade 3 contains specimens from upper Myanmar in Kachin State and Sagaing Region, Myanmar (Figure 6). Clade 5 includes samples from Tanintharyi Division, Malayan Peninsula, Myanmar. Clade 6 contains specimens from lower Myanmar (Yangon and Bago Regions). Clade 7 is distributed in the eastern portion of lower Myanmar (Kayin and Mon States) and northwestern Thailand (Mae Hong Son province).

Clade 7 includes topotypic specimens from the neotype locality of *L. limborgi* and are therefore considered to represent the "true" *L. limborgi*. Bioacoustical data are available for our Clades 6 and 7 (see also Table 5). The male advertisement calls of these two clades differ clearly (Figure 7): the call of males of "true" *L. limborgi* (Clade 7) consists of a series of three to five notes whereas males from Clade 6 emit a single "quaaak". The two calls differ also in the length of the notes (0.218–0.252 s, usually <0.250 s, in males of Clade 6 versus 0.241–0.419 s, usually >0.260 s, in males of Clade 7). The genetic distance (16S) between these two clades is 2.8% (Table 5).

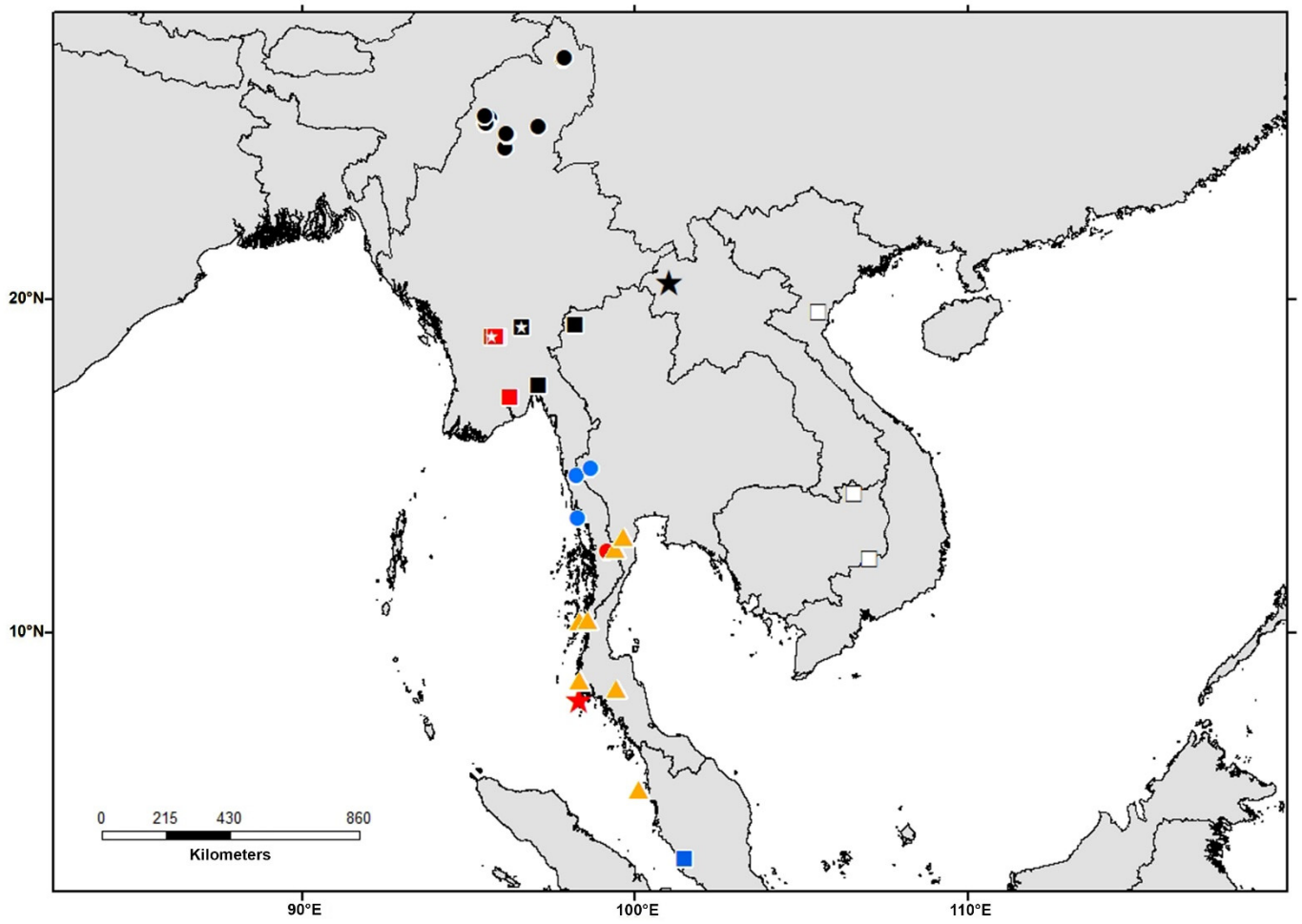

Figure 6. Map showing collecting localities of the *Limnonectes* species related to *L. limborgi*. Each symbol can represent one or more adjacent localities. Orange triangles = *L. hascheanus*; blue square = *L. limborgi* Clade 1; red circle = *L. limborgi* Clade 2; black circles = *L. limborgi* Clade 3; black star = *L. limborgi* Clade 4; blue circles = *L. limborgi* Clade 5; red squares = *L. limborgi* Clade 6; black squares = *L. limborgi* Clade 7; white squares = *L. limborgi* Clade 8; red star = *L. limborgi* Clade 9. Small white stars within symbols indicate localities from which bioacoustics data have been used in this study.

Table 5. Selected bioacoustic parameters of the species related to *Limnonectes limborgi*. Range is followed by mean value and standard deviation in parentheses. Dom. Freq. = dominant frequency; Freq. = frequency.

	Taxon ID	Call Duration (sec)	Dom. Freq (Hz)	Gap Duration (sec)	Number of Notes Per Call	Note Duration (sec)	Freq 5% (Hz)	Freq 95% (Hz)
GK-7209_0446 (n = 5 calls)	*L. limborgi*, Clade 7, Leiktho	1.13–1.58 (1.23 ± 0.18)	1335–1528 (1417 ± 29.2)	15.8–56.5 (33.3 ± 16.28)	3–4 (3.2 ± 0.40)	0.241–0.329 (0.274 ± 0.027)	904–1033 (986 ± 36.7)	1895–1981 (1951 ± 20.0)
GK-7209_0447 (n = 4 calls)	*L. limborgi*, Clade 7, Leiktho	1.57–2.87 (2.48 ± 0.53)	1550–1637 (1589 ± 24.1)	27.4–37.0 (32.2 ± 3.94)	3–5 (4.5 ± 0.87)	0.293–0.419 (0.336 ± 0.039)	1098–1163 (1120 ± 18.9)	2046–2132 (2099 ± 24.8)
GK-7110_0438 (n = 7 calls)	*L.* cf. *limborgi*, Clade 6, Bago Yoma	0.222–0.249 (0.235 ± 0.009)	1141–1464 (1307 ± 94.0)	21.5–64.6 (32.3 ± 3.3)	1	0.222–0.249 (0.235 ± 0.009)	753–797 (781 ± 15.0)	1830–1938 (1889 ± 45.6)
GK-7110_0439 (n = 9 calls)	*L.* cf. *limborgi*, Clade 6, Bago Yoma	0.218–0.252 (0.231 ± 0.010)	1335–1615 (1428 ± 78.6)	11.3–21.3 (16.1 ± 3.3)	1	0.218–0.252 (0.231 ± 0.010)	818–861 (846 ± 14.3)	1960–2046 (2007 ± 30.1)

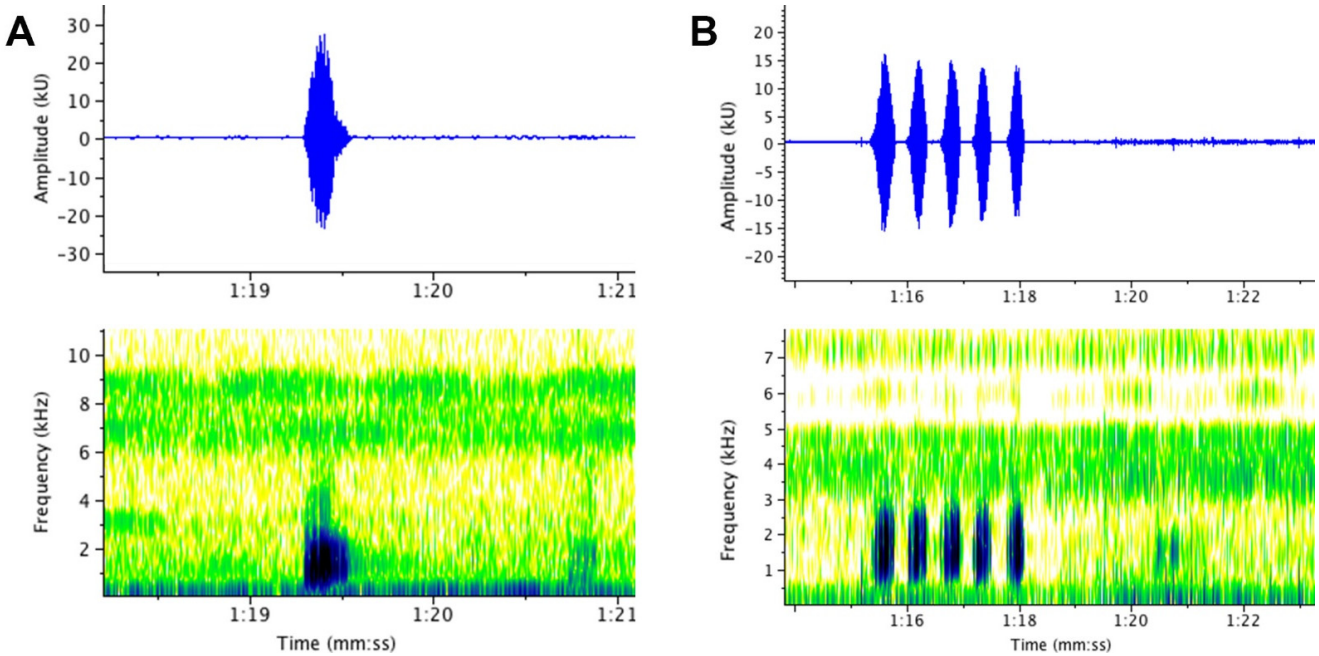

Figure 7. Advertisement calls of male *Limnonectes*. (**A**) *P. bagoyoma* n. sp., SMF 106035 (**B**) *L. limborgi*, GK-7209. See text for details and Figure 6 for localities plotted on map.

In external morphology, specimens of these five clades differ in details such as in the shape and orientation of the supratympanic fold, the development of the tarsal fold as well as in the amount of toe webbing, and in some details of coloration (see Table 3 and respective diagnosis sections below). Additionally, they show differences in some morphometric values (Figure 8), Finally, there is sexual dimorphism evident in several morphometric characters, most evident in relative head width.

Thus, based on the results of the integrative analyses of mtDNA data, morphology, and bioacoustics, we recognize Clades 6 and 7 as species-level units whereas the other clades remain candidate species awaiting further study, especially based on nuclear DNA data and bioacoustics. Based on the respective type locality, our Clade 7 is assigned to *Limnonectes limborgi* whereas our Clade 6 represents an undescribed species for which no name is available. Thus, we describe the *Limnonectes* related to *L. limborgi* from lower Myanmar (Yangon and Bago Regions) as a new species below.

This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the International Commission on Zoological Nomenclature (ICZN). The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information can be viewed through any standard web browser by appending the LSID to the prefix http://zoobank.org. The LSID for this publication is as follows: urn:lsid:zoobank.org:pub:4C463126-CD59-4935-96E3-11AF4131144C. The LSID registration and any associated information can be viewed in a web browser by adding the LSID to the prefix "http://zoobank.org/".

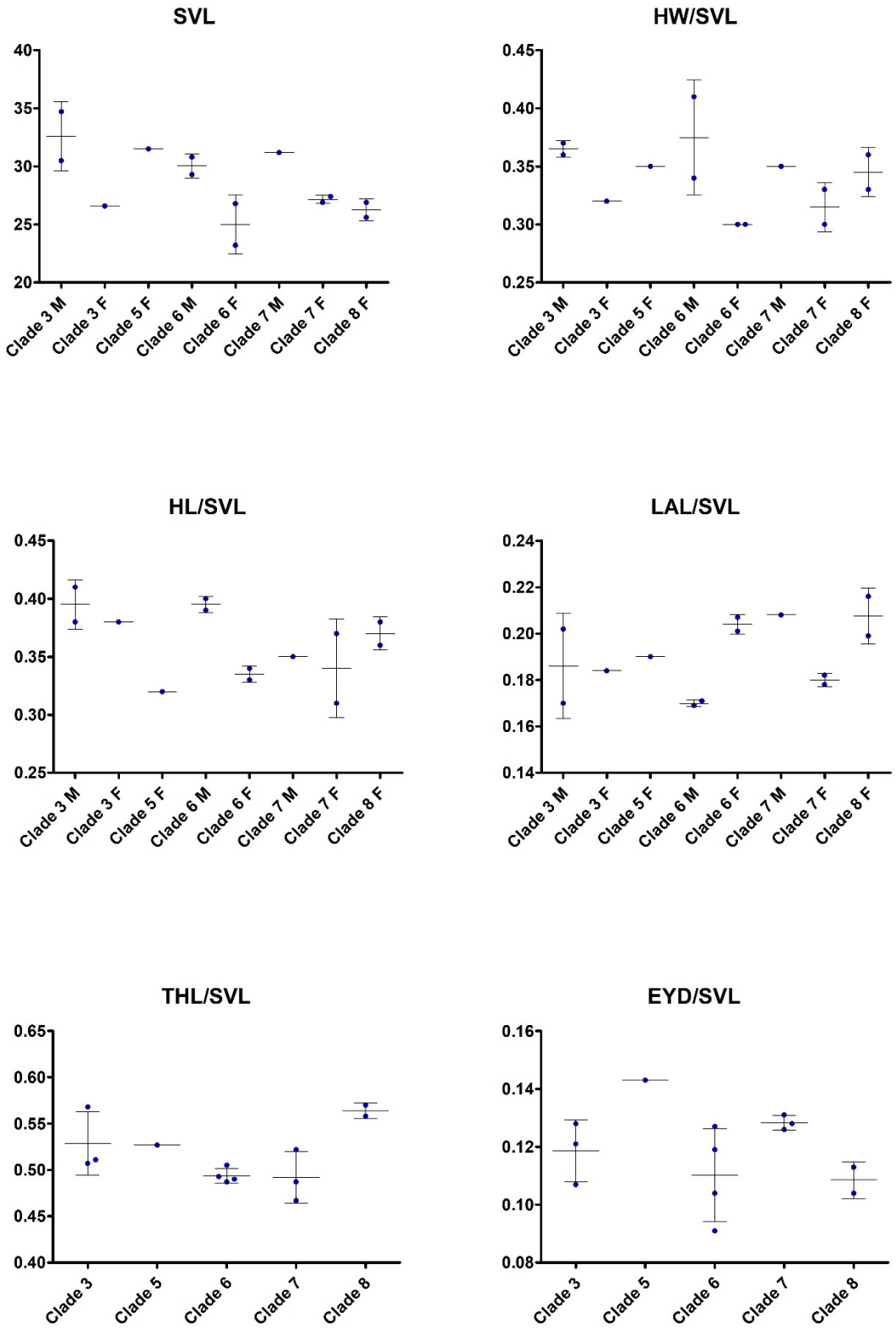

Figure 8. *Cont*.

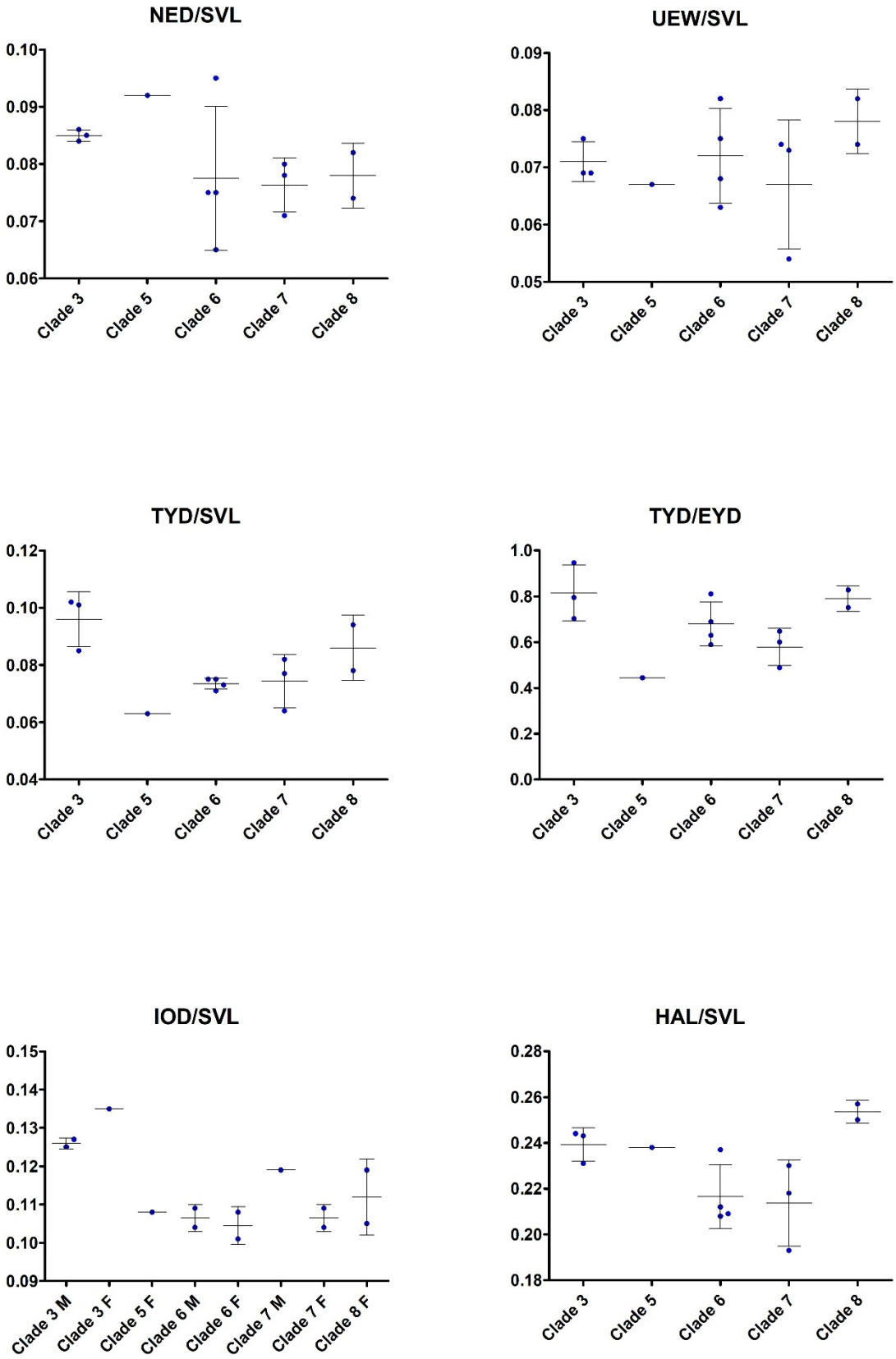

Figure 8. Scatter plots illustrating morphological variation in the clades frogs related to *Limnonectes limborgi*. M = males; F = females. For definitions of Clades and abbreviations of morphological characters see text.

***Limnonectes bagoensis* sp. nov.**

Gunther Köhler, Britta Zwitzers, Ni Lar Than and Panupong Thammachoti

urn:lsid:zoobank.org:act:5EA582A8-39DB-4259-A8D9-4C8FADAC5E9A

Holotype. SMF 104090, an adult male collected in Bago, near the airport (17.32922, 96.44025; 35 m a.s.l.), Bago Region, Myanmar, collected 7 July 2017 by Gunther Köhler and Ni Lar Than. Field tag number GK-6742.

Paratypes. All Bago or Yangon Region, Myanmar. SMF 104091 (field tag number GK-6743), adult female, same collecting data as holotype. SMF 106038 (field tag number GK-7091), adult male, Bago Yoma (18.89255, 95.69519; 135 m a.s.l.), collected 16 June 2019 by Gunther Köhler and Ni Lar Than. 106039 (field tag number GK-7093), adult male, Bago Yoma (18.89854, 95.87848; 420 m a.s.l.), collected 16 June 2019 by Gunther Köhler and Ni Lar Than. SMF 106040-41 (field tag numbers GK-7142, 7147), adult females, Bago Yoma (18.89410, 95.88054; 405 m a.s.l.), collected 20 June 2019 by Gunther Köhler and Ni Lar Than. SMF 106042-43 (field tag numbers GK-7147, 7148), adult females, Bago Yoma (18.90105, 95.87756; 440 m a.s.l.), collected 20 June 2019 by Gunther Köhler and Ni Lar Than. CAS 213272, adult male, Mingalardon Township, Hlawga Wildlife Park (17.04656, 96.11078), Yangon Region, Myanmar, collected 17 December 1999 by T. Thin, S. W. Kyi and Y. M. Win.

Referred specimens. **Myanmar:** Bago: Bago Yoma (18.910667, 95.801350; 250 m a.s.l.): HML-30, 33.

Diagnosis. A species of the genus *Limnonectes* to which it is assigned because of its inferred phylogenetic position, males with hypertrophied heads, and the presence of odontoids on the lower jaw in adult males [19,57]. *Limnonectes bagoensis* sp. nov. is assigned to the subgenus *Elachyglossa* [17] because of its close phylogenetic position to the type species of this subgenus (i.e., *L. gyldenstolpei*). *Limnonectes bagoensis* sp. nov. differs from all congeners by having (1) a medium body size (males 32–49 mm; females 30–47 mm); (2) slightly enlarged toe disks; (3) adult males with small odontoids in the lower jaw; (4) adult males without a knob-like or flap-like structure (caruncle) in the interorbital and parietal region; also lacking swelling in the occipital region; (5) in adult males head not conspicuously enlarged; (6) webbing formula I 1–2 II 1–2.4 III 1.2–3 IV 3.3–2 V to I 1.8–2.2 II 1.5–2.8 III 2.2–3.2 IV 3.5–2 V; (7) a feeble dermal fringe along the outer edge of Toe 5; (8) male advertisement call consists of two portions that are separated by a break of slightly less than 1 s. The first portion of the call is a single note with a duration of 225–291 ms whereas the second portion consists of 4–9 notes with a duration of 1200 (4 notes) to about 2000 ms (9 notes) with about 4.5 notes per second; the dominant frequency is at 861–990 Hz (mean 945 Hz). *Limnonectes bagoensis* differs from all other species currently assigned to the subgenus *Elachyglossa* except *L. doriae* by lacking a knob-like or flap-like structure (caruncle) in the interorbital and parietal region in adult males (versus such structure present in adult males), and also lacking swelling in the occipital region (versus such swelling present in adult males). *Limnonectes bagoensis* differs from *L. doriae* in having a male advertisement call that consists of two portions, separated by a break of slightly less than 1s, the second portion with a series of 4–9 notes at about 4.5 notes per second (versus a single series of notes in *L. doriae* with about 12 notes per second); gular coloration in adult males not dark (versus gular region dark gray in adult males of *L. doriae*).

Description of the holotype (Figure 9). Adult male, as indicated by the presence of odontoids, broad head, and folds along mandibular arch; SVL 40.8 mm; habitus robust; head broad, about as wide as long, ratio HL/HW 1.07; snout nearly rounded in dorsal view, projecting beyond lower jaw, rounded in profile; nostril dorsolateral, closer to tip of snout than eye; canthus rounded; ratio EYD/SVL 0.10; IOD (4.8 mm) greater than width of upper eyelid (3.1 mm); tympanum distinct, slightly recessed relative to skin of temporal region, tympanic rim distinctly elevated relative to tympanum; ratio TYD/EYD 0.85; four to five vomerine teeth on slightly raised oblique ridges between choanae, separated from choanae by about 1/4 length of one group, gap between groups about 1/2 length of one group; tips of all four fingers rounded, not expanded into discs; relative finger lengths III > I > IV > II; no webbing; distinct subarticular tubercles; supernumerary tubercles ab-

sent; palmar tubercle distinct, flat, bifid; thenar tubercle large, elongate, raised, about twice the size of palmar tubercle; tips of toes rounded, slightly expanded into discs; relative toe lengths IV > III > V > II > I; feet almost fully webbed, webbing formula I 1–1.5 II 0.5–2 III 1–2.5 IV 2.5–1 V; a well-developed dermal ridge along lateral side of Toe V from level of outer metatarsal tubercle to distal subarticular tubercle; large, flap-like inner metatarsal tubercle, >0.5 length of first toe; distinct fold on distal one-half of tarsus beginning at inner metatarsal tubercle; outer metatarsal tubercle tiny; skin on dorsal and lateral surfaces of head, body, and limbs smooth except for a few scattered tubercles in sacral region and on dorsal surface of shank; skin on throat and venter smooth; distinct supratympanic ridge from posterior edge of upper eyelid along upper margin of tympanum and then obliquely down to shoulder; no dorsolateral fold. Measurements (mm) of holotype: SVL 40.8; HL 17.4; HW 16.3; SL 6.6; EYD 4.1; IOD 4.8; TYD 3.5; TED 1.7; SHL 22.1; THL 20.0; HNL 10.2; FL 20.4; NED 4.2; IND 4.6.

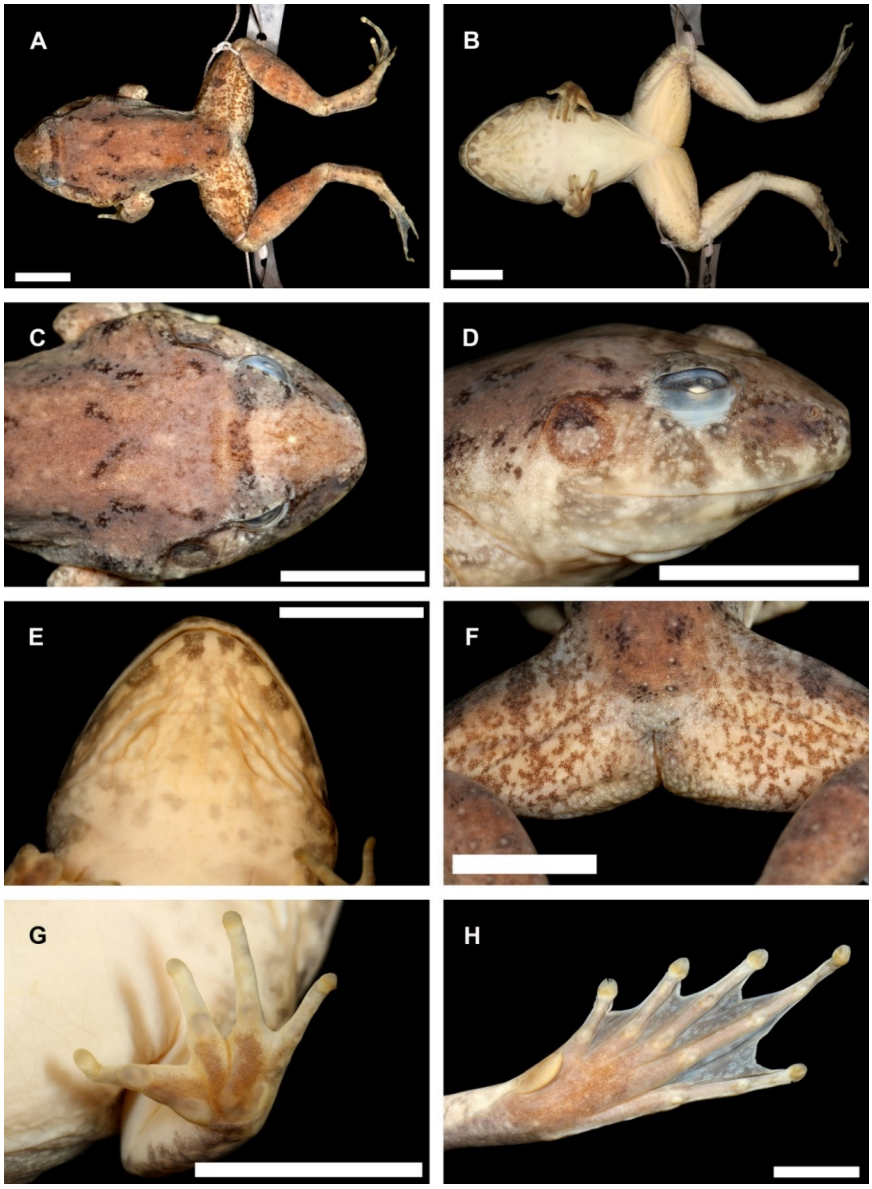

Figure 9. Holotype of *Limnonectes bagoensis* n. sp. (SMF 104090). (**A**). dorsal view; (**B**). ventral view; (**C**). dorsal view of head; (**D**). lateral view of head; (**E**). ventral view of head; (**F**). cloacal and thigh region; (**G**). palmar surface of hand; (**H**). plantar surface of foot. Scale bars equal 5.0 mm. Photos by G.K.

Coloration in life was recorded as follows (Figure 10A): Dorsal surfaces of head and body Olive Horn Color (16) with a suffusion of Smoke Gray (267) in the occipital region and with a Dark Drab (45) interorbital bar; dorsum with a few Mikado Brown (42) and Grayish Olive (273) splotches; labial region Light Buff (2) with prominent broad Dark Drab (45) vertical bars; dorsal surfaces of hind limbs Pale Cinnamon (55) with Olive-Brown (278) transverse bars; iris Buff (5) above and whitish below and with a Sepia (279) streak in upper and lower edge as well as a suffusion of Grayish Olive (274) in lateral portions.

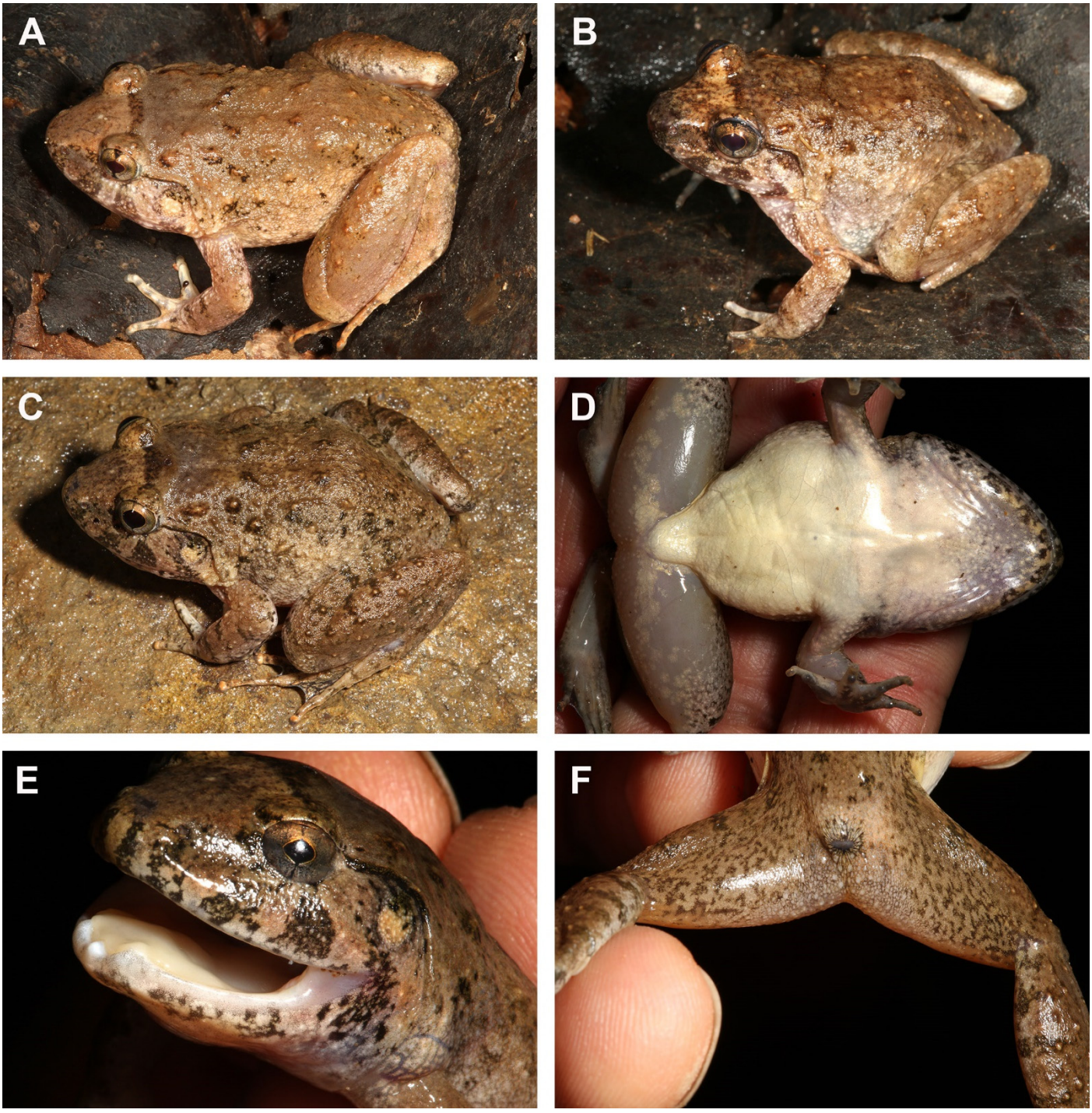

Figure 10. *Limnonectes bagoensis* n. sp. in life. (**A**) Male holotype, SMF 104090; (**B**) female paratype, SMF 104091; (**C–F**) male paratype, SMF 106038. Photos by G.K.

Coloration after about three and half years preservation in 70% ethanol was recorded as follows: Dorsal and lateral ground color of head and body Burnt Umber (48) with broken Sepia (286) transverse zigzag line across anterior dorsum and with a Sepia (286) interorbital bar; labial region Light Buff (2) with prominent broad Sepia (279) vertical bars; dorsal surfaces of hind limbs Grayish Horn Color (268) with Olive-Brown (278) transverse bars; ventral surfaces of head, body, and limbs Light Buff (2) with indistinct Smoke Gray (266) mottling in the gular region; palmar surfaces Ground Cinnamon (270); plantar surfaces Fawn Color (258).

Variation. The paratype and the referred specimens agree well with the holotype in general appearance, morphometrics, and coloration. The coloration of an adult male (SMF 106038; Figure 10C–F) from Bago Yoma was noted as follows: Dorsal background color of head and body Olive-Brown (278) with Sepia (286) splotches on dorsum and with a Raw Umber (280) interorbital bar, edged anteriorly by a Clay Color (20) bar; labial region Clay Color (18) with broad Sepia (279) vertical bars; tympanum Clay Color (20); occipital region suffused with Hair Brown (277); flank region Chamois (84); dorsal surfaces of hind limbs Olive-Brown (278) with Sepia (286) transverse bars; tips of fingers and toes Salmon Color (83); posterior surface of thigh Tawny Olive (17) with Olive-Brown (278) mottling; ventral surfaces of head and body Whitish Lime Green (111) with a suffusion of Sulphur White (96) on posterior body; area adjacent to mandibular arch Medium Neutral Gray (298) with Whitish Lime Green (111) speckles; ventral surfaces of limbs Hair Brown (277) with Sulphur White (96) splotches; palmar and plantar surfaces suffused with Sepia (279); iris Drab (19) with a suffusion of iris Tawny Olive (17) in upper portion.

Etymology. The species name "*bagoensis*" refers to the city of Bago where the holotype of this species was collected, and *ensis* denoting place.

Natural history notes. At the type locality at night time, adult males were heard calling on the surface hidden under flat rocks. Figure 11 shows the holotype (SMF 104090) that continued calling even after the covering rocks had been removed. Additional individuals were encountered sitting on the forest floor at night without cover.

Figure 11. Holotype of *Limnonectes bagoensis* n. sp. (SMF 104090) in life, at its calling site after the covering rocks had been removed.

Geographic Distribution and Conservation. As currently known, *Limnonectes bagoensis* sp. nov. is known from several localities in Bago Region, Myanmar. Given how little we know about this species, we classify *Limnonectes bagoensis* sp. nov. as Data Deficient based on the IUCN Red List Categories and Criteria [58].

Genomic characterization. Whole-genome sequencing and assembly (Table 6): Illumina sequencing produced 302,829,670 short-read pairs with a total sequence amount of 90 Gb. K-mer analysis estimated the genome size of 2.16 Gb with 0.78% heterozygosity. (Figure 14) The mitochondrial genome was assembled into one 17,480 bp long circular contig. A total of 13 protein-coding genes, two rRNAs, 22 tRNAs were annotated in the mitochondrial genome (Figure 12). A D-loop region could not be annotated in the mitochondrial genome sequence.

Table 6. Genomic details of the holotypes of the two new species are described herein.

	SMF 104090	SMF 106034
Raw read pairs (Illumina)	302,829,670	278,081,641
Read length	150	150
Total bases	90,848,901,000	83,424,492,300
Survived Reads (%)	93.5	92.3
Heterozygosity (%)	0.78	0.78
Assembled Nuclear Genome	1,941,736,683	2,099,953,831
Number of Scaffolds	647,264	691,036
Scaffold N50	4270	4236
Largest Scaffold	42,771	42,830
Smallest Scaffold	500	500
GC%	34.74	42.54
Circular Mito-Genome	17,480 bp	17,604 bp

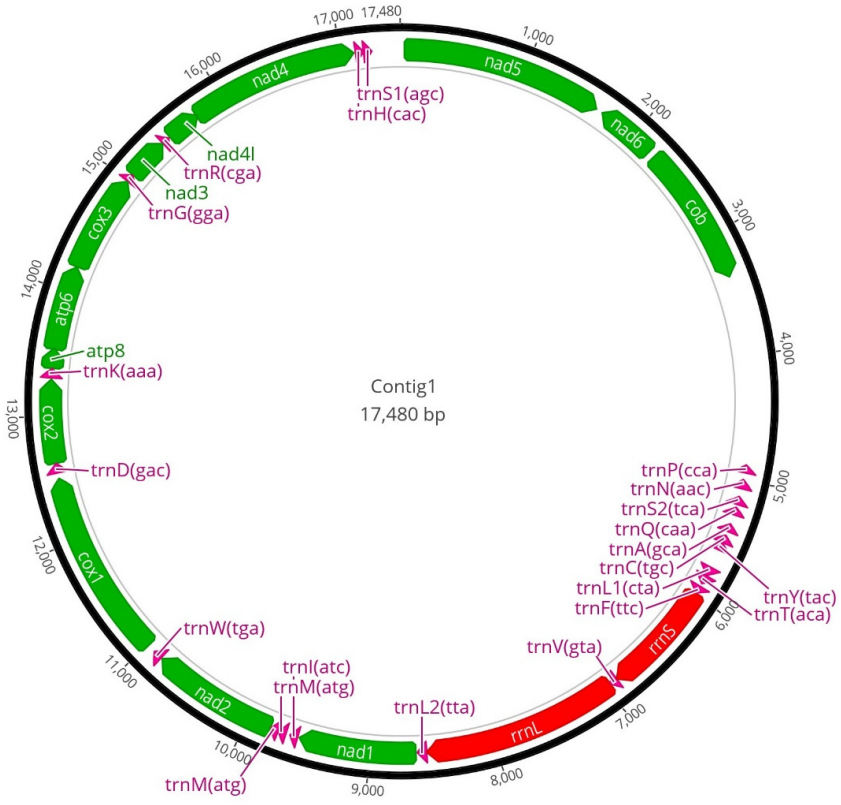

Figure 12. Annotated mitochondrial genome of the holotype of *Limnonectes bagoensis* n. sp. (SMF 104090) see text for details.

The nuclear genome obtained by reference-based assembly consisted of 1,941,736,683 bases fragmented in 647,264 scaffolds. The longest scaffold was 42,771 bases and the N50 scaffold was 4270 bases whereas GC content was only 34.74%. Except for the high-quality mitochondrial genomes, the simple assembly from Illumina short-reads was predicted to be 26.2% complete as per the BUSCO gene space [59] (C:12.4%(S:12.3%, D:0.1%), F:13.9%, M:73.7%, n:5310). Raw reads and the draft-genome assemblies can be found within the BioProject PRJNA745544.

***Limnonectes bagoyoma* sp. nov.**

Gunther Köhler, Britta Zwitzers, Ni Lar Than and Panupong Thammachoti

urn:lsid:zoobank.org:act:412732A4-47E3-4032-8FF0-7518BA232F9F

Holotype. SMF 106034, an adult female collected in Bago Yoma (18.89255, 95.69519; 135 m a.s.l.), Bago Region, Myanmar, collected 16 June 2019 by Gunther Köhler and Ni Lar Than. Field tag number GK-7110.

Paratypes. All paratypes were collected in Bago Yoma, Bago Region, Myanmar. SMF 106035, adult male, same collecting data as holotype; SMF 106036, adult female, collected 20 June 2019 by Gunther Köhler and Ni Lar Than at 18.89876, 95.87889; 435 m a.s.l. SMF 106037, adult male, collected 20 June 2019 by Gunther Köhler and Ni Lar Than at 18.90128, 95.87737; 440 m a.s.l.

Referred specimens. **Myanmar:** Bago: Bago Yoma (18.910667, 95.801350; 250 m a.s.l.): HML-32.

Diagnosis. A species of the genus *Limnonectes* to which it is assigned because of its inferred phylogenetic position (Figure 1), males with hypertrophied heads, and the presence of odontoids on the lower jaw in adult males [19,57]. *Limnonectes bagoyoma* sp. nov. is assigned to the subgenus *Taylorana* because of its close phylogenetic position to the type species of this subgenus (i.e., *L. limborgi*; Figure 1). *Limnonectes bagoyoma* sp. nov. differs from all congeners by having (1) a small body size (males 29.3–30.7 mm; females 23.1–26.6 mm); (2) males with slightly enlarged odontoids; (3) inner metatarsal tubercle large, raised, usually >0.5 length of the first toe; (4) webbing formula I 1–2 II 1–2.4 III 1.2–3 IV 3.3–2 V to I 1.8–2.2 II 1.5–2.8 III 2.2–3.2 IV 3.5–2 V; (5) females with clutches of enlarged, non-pigmented eggs; (6) male advertisement call consisting of a single note with a duration of 220–250 ms and a dominant frequency mostly in the 1300–1400 Hz range. *Limnonectes bagoyoma* differs from the remaining species of the subgenus *Taylorana* (see also [15]) as follows: from *L. limborgi* by having a male advertisement call consisting of a single note with a duration of 220–250 ms (versus call consisting of a series of 3–5 notes, each note with a duration of usually >260 ms in *L. limborgi*), and no dark lateral face mask (versus dark face mask present). *Limnonectes bagoyoma* differs from *L. hascheanus* by its larger body size, reaching 30 mm SVL in adult males (versus SVL <26 mm in both sexes in *L. hascheanus*), and by having more toe webbing, usually three phalanges free of webbing on the medial side of Toe 4 (versus more than three phalanges free of webbing on the medial side of Toe 4). *Limnonectes bagoyoma* differs from *L. liui* by its smaller body size, SVL not exceeding 31 mm in both sexes (versus SVL 32.0–38.5 mm in males of *L. liui*, only female in type series measuring 32.7 mm), toe webbing well-developed (versus rudimentary), and inner metatarsal tubercle usually >0.5 length of the first toe (versus "just half" of the first toe in *L. liui* according to [15]). *Limnonectes bagoyoma* differs from *L. medogensis* by having the nostril positioned closer to the snout than to the eye (versus closer to eye in *L. medogensis*), vomerine teeth present (versus absent), venter patternless (versus marbled markings present), toe webbing well-developed (versus rudimentary), inner metatarsal tubercle distinctly shorter than the first finger (versus about length of Toe 1), and tarsal fold indistinct but present (versus absent). *Limnonectes bagoyoma* differs from *L. xizangensis* by having the toe tips enlarged into small disks (versus toe tips pointed), vomerine teeth present (versus absent), toe webbing well-developed (versus absent), and the presence of tiny tubercles on the dorsal surface of the shank (versus smooth), venter patternless (versus black reticulated markings present), and smooth skin on venter (granular skin on venter). *Limnonectes bagoyoma* differs from *L. alpinus* by having the toe webbing well-developed

(versus toe webbing absent in *L. alpinus*), vomerine teeth present (versus absent), and tympanum distinct (versus hidden).

Description of the holotype (Figure 13). Adult female, as indicated by the rather slender head, absence of odontoids, and absence of folds along mandibular arch; SVL 26.8 mm; habitus robust; head narrow, slightly longer than wide, ratio HL/HW 1.14; snout subovoid in dorsal view, projecting beyond lower jaw, rounded in profile; nostril dorsolateral, closer to tip of snout than eye; canthus rounded; ratio EYD/SVL 0.13; IOD (2.7) greater than width of upper eyelid (1.8 mm); tympanum distinct, slightly recessed relative to skin of temporal region, tympanic rim distinctly elevated relative to tympanum; ratio TYD/EYD 0.59; four to five vomerine teeth on slightly raised oblique ridges between choanae, separated from choanae by about 1/2 length of one group, gap between groups about 1/2 length of one group; tips of all four fingers rounded, not expanded into discs; relative finger lengths III>I>II>VI; no webbing; distinct subarticular tubercles; supernumerary tubercles absent; palmar tubercle distinct, flat, bifid; thenar tubercle large, elongate, raised, about twice the size of palmar tubercle; tips of toes rounded, slightly expanded into discs; relative toe lengths IV>III>V>II>I; feet almost fully webbed, webbing formula I 1.5–2 II 1.5–2.5 III 2.2–3 IV 3.5–2 V; a weakly developed dermal ridge along lateral side of Toe V from level of outer metatarsal tubercle to distal subarticular tubercle; large, flap-like inner metatarsal tubercle, >0.5 length of first toe; outer metatarsal tubercle tiny; tarsal fold indistinct; skin on dorsal and lateral surfaces of head, body, and limbs smooth except for a few scattered tiny tubercles in sacral region and on dorsal surface of shank; skin on throat and venter smooth; indistinct supratympanic ridge from posterior edge of upper eyelid along upper margin of tympanum and then obliquely down to shoulder; no dorsolateral fold. Measurements (mm) of holotype: SVL 26.8; HL 9.2; HW 8.1; SL 3.8; EYD 3.4; IOD 2.7; TYD 2.0; TED 1.3; SHL 14.0; THL 13.2; HNL 9.2; FL 12.7; NED 2.0; IND 2.5.

Coloration in life was recorded as follows (Figure 14A): Dorsal ground color of the head and body Dark Salmon Color (59) with a suffusion of Light Orange Yellow (7) on the dorsal head in front of broken Burnt Umber (48) interorbital bar; a Raw Umber (280) band below canthus rostralis and along the supratympanic ridge; flank region suffused with Buff-Yellow (6); dorsal surfaces of limbs Orange-Rufous (56) with Brownish-Olive (276) transverse bars; iris Cream Color (12) in the upper portion, Grayish Horn Color (268) below and with a suffusion of Warm Sepia (40) in anterior and posterior corners.

Coloration after about one and half years preservation in 70% ethanol was recorded as follows: Dorsal and lateral ground color of the head and body Drab (19), but paler, with a suffusion of Smoke Gray (266) on the dorsal head and with indistinct Vandyke Brown (281) stipples on dorsum and as a broken interorbital bar as well as a band below canthus rostralis and along the supratympanic ridge; Glaucous (289) vertical bars on the lower lip; dorsal surfaces of the thigh and shank Drab (19) with indistinct Glaucous (289) transverse bars; ventral surfaces of head, body, and limbs Cream White (52); palmar surfaces Ground Cinnamon (270); plantar surfaces Natal Brown (49).

Variation. The paratypes agree well with the holotype in general appearance; morphometrics and coloration (see Table 3). The two adult paratype males both have broad heads, folds along the mandibular arch, distinct odontoids, and four to five vomerine teeth. The coloration of a topotypic adult male (SMF 106035; Figure 14B) was noted as follows: Dorsal surface of head Pale Pinkish Buff (3) with Natal Brown (49) streaks; loreal region Cinnamon-Drab (50); a Raw Umber (280) band below canthus rostralis and along the supratympanic ridge; body Salmon Color (83) above and grading into Chamois (84) on the flank; dorsal surfaces of limbs Olive-Brown (278) with Hair Brown (277) transverse bars; iris Tawny Olive (17) in the upper portion, Grayish Horn Color (268) below and with a suffusion of Warm Sepia (40) in anterior and posterior corners.

Etymology. The species name "*bagoyoma*" refers to the type locality Bago Yoma, a large but relatively low mountain range that runs in a north-south direction between the Irrawaddy (=Ayeyarwady) and the Sittaung River in Myanmar. It is likely that *Limnonectes bagoyoma* is geographically restricted to this mountain range.

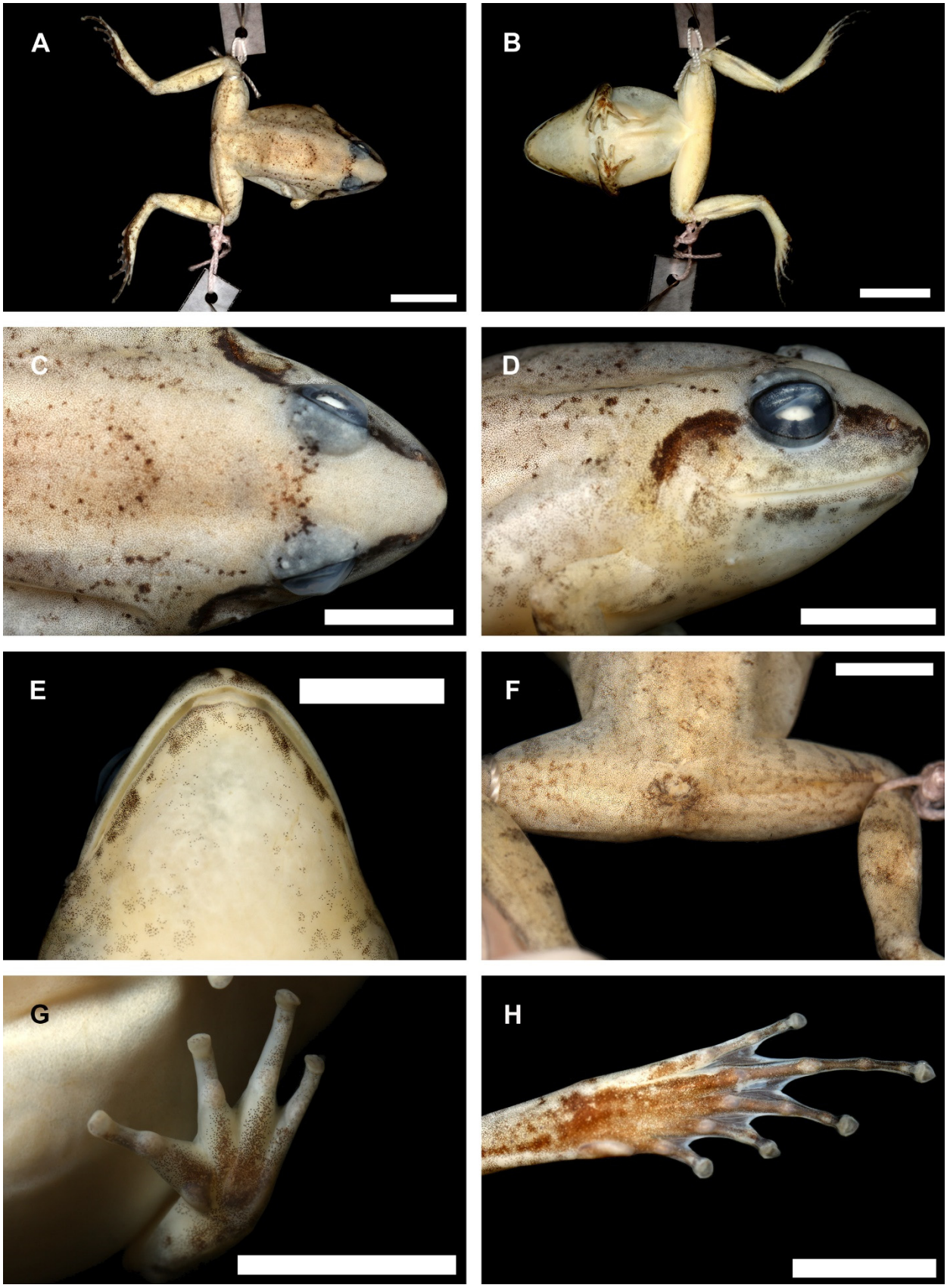

Figure 13. Holotype of *Limnonectes bagoyoma* n. sp. (SMF 106034). (**A**). dorsal view; (**B**). ventral view; (**C**). dorsal view of head; (**D**). lateral view of head; (**E**). ventral view of head; (**F**). cloacal and thigh region; (**G**). palmar surface of hand; (**H**). plantar surface of foot. Scale bars equal 5.0 mm. Photos by G.K.

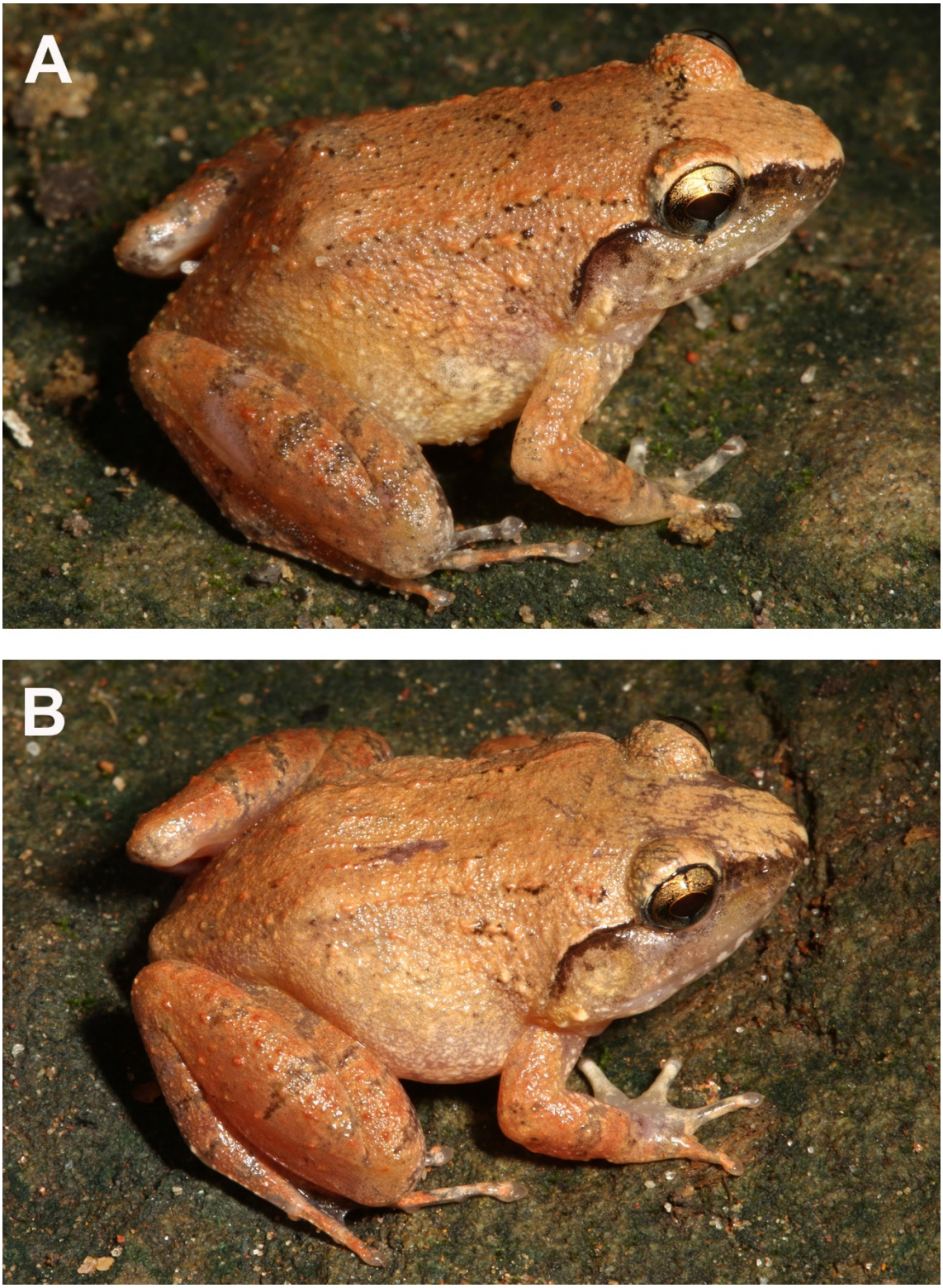

Figure 14. *Limnonectes bagoyoma* n. sp. in life. (**A**) Female holotype, SMF 106034; (**B**) Male paratype, SMF 106035. Photos by G.K.

Natural history notes. At the type locality, the specimens were collected during the daytime in leaf litter in the rainforest. The two males we collected were heard calling before

we caught them. Each male was sitting in a shallow depression, covered by leaf litter. Other frog species collected at the type locality include *Duttaphrynus melanostictus, Fejervarya orissaensis, Ingerana tenasserimensis, Limnonectes bagoensis,* and *Leptobrachium smithi*.

Geographic Distribution and Conservation. As currently known, *Limnonectes bagoyoma* sp. nov. is only known from the vicinity of its type locality. Given the little we know about this species, we classify *Limnonectes bagoyoma* sp. nov. as Data Deficient based on the IUCN Red List Categories and Criteria [58].

Genomic characterization. Whole-genome sequencing and assembly (Table 6): Pacbio sequencing resulted in 16,749,860 reads of size 90 Gb whereas Illumina sequencing produced 278,081,641 short read pairs with a total sequence amount of 83.4 Gb. K-mer analysis estimated the genome size of 2.4 Gb with 0.78% heterozygosity (Figure 15). A mitochondrial genome of 17,406 bases was assembled using Pacbio reads which matched to the complete annotated mito-genome of the holotype SMF 104090. The annotation resulted in 13 protein-coding genes, three rRNAs, 22 tRNAs and three replication origin sites. A D-loop region was also annotated in the mitochondrial genome sequence. (Figure 16) .

The draft nuclear genome assembly contains 691,036 scaffolds with a total length of 2,099,953,831 bps, an N50 of 4,236 bps and a GC content of 42.54%. The BUSCO [57] search allowed to identify 31.1% of the BUSCO genes [59] (C:13.2% [S:13.1%, D:0.1%], F:17.9%, M:68.9%, n:5310). Raw reads and the draft-genome assemblies can be found within the BioProject PRJNA745544.

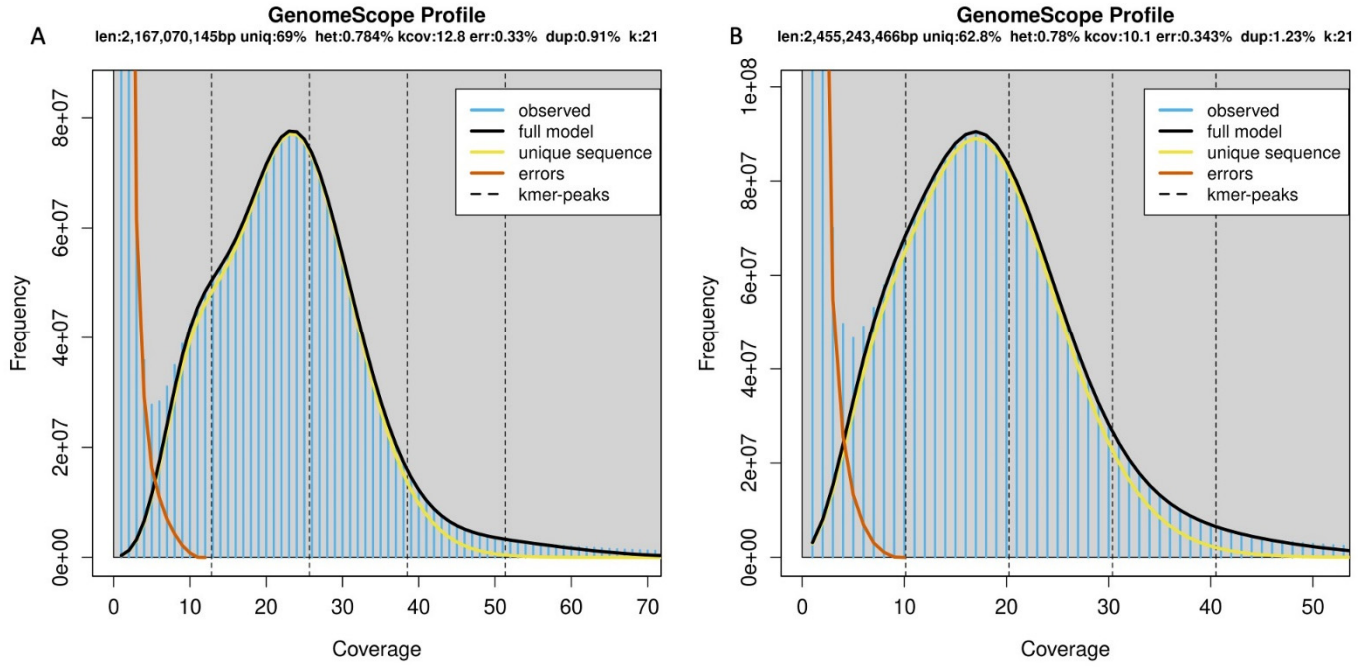

Figure 15. Results of K-mer analysis. See text for details.

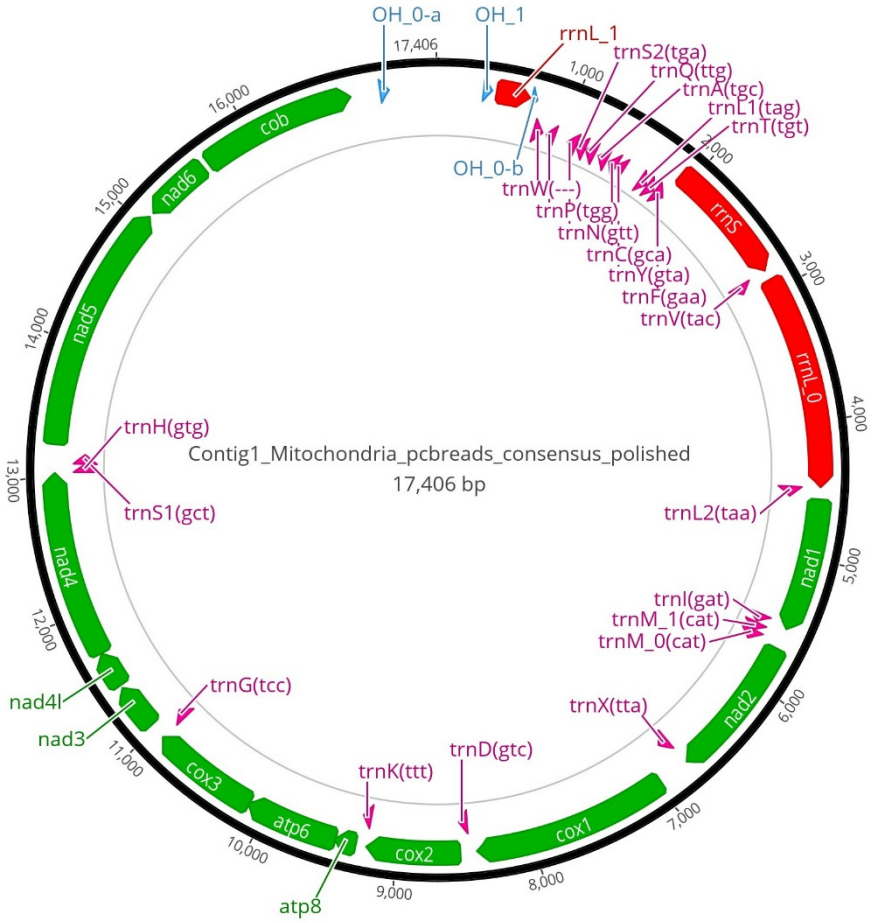

Figure 16. Annotated mitochondrial genome of the holotype of *Limnonectes bagoyoma* n. sp. (SMF 106034) see text for details.

4. Discussion

The present study presents further examples for putative geographically wide-spread species that actually represent species complexes of several taxa that are similar in external morphology but distinct in bioacoustics and genetics ([5,21,60]). To resolve the diversity in these complexes, additional field research is needed to obtain fresh voucher specimens from across their range for genetic and bioacoustics characterization of the geographically distinct populations. The case of *Limnonectes bagoensis* reported here, shows that in some cases the genetic differentiation in mtDNA can be rather low (i.e., just 2% in 16S between *L. bagoensis* and its putatively most closely related sister species *L. doriae*). However, given their rather drastically different male advertisement calls, there is no doubt that these two forms are distinct species. This hypothesis is reinforced by the observation that the male advertisement call of *L. bagoensis* does not vary across a large geographic distance (i.e., almost 200 air-line km). In the case of the species pair *L. limborgi* and *L. bagoyoma*, the genetic differentiation in mtDNA data (16S) is close to 3%, and the species are distinct in bioacoustics, too. In the course of this study, we identified additional candidate species in either species complex. Due to the lack of additional evidence lines we refrained from naming any of these groups defined only by mtDNA data. For "*L. limborgi*" from northern Vietnam (which corresponds with our Clade 10) the male advertisement call has been described as "single notes, repeated in very large intervals from 30 s to several minutes", an observation confirmed by [61]. The latter author reports the call to have a dominant frequency of 1000–1100 Hz and a duration of 278–331 ms. Both accounts describe the male sitting in a hole with just the head visible after the covering leaves had been removed. Except for some details, this call description agrees very well with our analyses of male advertisement calls of *L. bagoyoma* and is unlike the call of the geographically closer *L.*

limborgi from the neotype locality of the latter species. Both the Vietnamese "*L. limborgi*" and *L. bagoyoma* have a male advertisement call consisting of a single note as opposed to a series of 3–5 notes in "true" *L. limborgi*. The calls of males from Vietnam have a dominant frequency of 1000–1100 Hz and a duration of 278–331 ms versus a dominant frequency mostly in the 1300–1400 Hz range and a duration of 220–250 ms in *L. bagoyoma*. The genetic distance in 16S between these two clades with similar calls (i.e., our Clades 6 and 10) is 3.2% whereas it is 2.5% between the Vietnamese "*L. limborgi*" and "true" *L. limborgi*. In order to determine whether the two similar calls are cases of convergence or have both remained in the ancestral state, it would be essential to know which call type represents the plesiomorphic and which one the apomorphic character state. A more robust genetic phylogeny, including nuclear markers as well as bioacoustical data from additional populations of this species complex, is needed to gain a better understanding of call evolution in this group of frogs. In frogs, bioacoustic data and analyses are as useful as are molecular genetics and often yield initial information on the presence of cryptic taxonomic diversity [60,62,63]. The male advertisement call in frogs works as an effective isolating mechanism to avoid hybridization among similar species under natural conditions [28].

With this description of two new species, we provide the complete, annotated mitochondrial genome as well as a draft genome of short-read genome resources of the holotypes of both species described herein. This genomic characterization of the species based on the name-bearing specimen will be a genomic resource for any forthcoming studies.

Author Contributions: Conceptualization, G.K.; methodology, G.K., P.T. and D.K.G.; Marker-based analysis, G.K., B.Z. and P.T.; bioinformatics genomic data, D.K.G.; fieldwork and specimen acquisition, G.K., N.L.T. and P.T.; writing—original draft preparation, G.K.; writing—review and editing, all authors; funding acquisition, A.J., S.U.P. and P.T. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Chulalongkorn University Animal Care and Use Committee of Chulalongkorn University, Bangkok (protocol code 2123010; 1 January 2021).

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A. Comparative Specimens Examined

Limnonectes bagoensis n. sp.—**Myanmar:** Bago: Bago, near airport: SMF 104090–91; Bago Yoma: HML-30, 33; SMF 106038-43.

Limnonectes bagoyoma n. sp.—**Myanmar:** Bago: Bago Yoma: HML-32, SMF 106034-37.

Limnonectes doriae—**Myanmar:** Mawlamyine: Paung Township: SMF 104084–89; Mon State: Kyaikto Township, along the trail from Kinmon to Kyaikto Pagoda: CAS 240316; Rakhine State: Rakhine Yoma Mountain Range: CAS 211632–33; Tanintharyi: Bokpyin Township, Pakchan Reserve Forest, Main Ma The camp: CAS 229581; Khamaukgyi Township: CAS 247792, 247851; Khamaukgyi Township, Kawthaung-Bokpyin road: CAS 247238–39; Thayetchaung Township, between Mal Ke Village and Ye Bya Village: CAS 229769; Thayetchaung Township, E Mal Ke Village, Nwalabo Reserve Forest: CAS 232304; Yebyu Township, Tanintharyi Nature Reserve: CAS 247908–09.

Limnonectes *hascheanus*—**Myanmar:** Tanintharyi: Khamaukgyi Township: CAS 247853; Khamaukgyi Township, Khamaukgyi Creek: CAS 247245.

Limnonectes limborgi—**Myanmar:** Kachin State: Machanbaw Township, the road between Ahtonga and Babaw: CAS 221462; Machanbaw Township, between Ahtonga and Aureinga: CAS 221424; Mohnyin Township, Indawgyi Wildlife Sanctuary, Nankan Forest Reserve, Mine Naung Village: CAS 228510–12, 228515, 228519; Nagmung Township, Au Yin Ga camp: CAS 225209; Kayin: Leiktho: GK-7209–10, 7216; Mon State: Kyaikto Township, Kinpon Chaung village, along waterfall stream: CAS 240382–83; Tanintharyi: Yebyu Township, Tanintharyi Nature Reserve: CAS 247950. **Thailand:** Kanchanaburi: 20 km N Tha Kah-nun: PT0236–37; Mae Hong Son: near Ban Nam Rin: Kovac_3a, Kovac_6; Phuket: near Kathu: PT1597.

Appendix B

Table A1. GenBank Accession Numbers of Genetic Sequences (16S) Used.

Species	GenBank Accession No.	Voucher
Fejervarya limnocharis	AF206466	USNM 520407
Limnonectes bagoensis	MZ578030	HML-030
Limnonectes bagoensis	MZ578031	HML-033
Limnonectes bagoensis	MZ578010	CAS 213272
Limnonectes bagoensis	MZ578025	GK-6742/SMF 104090
Limnonectes bagoensis	MZ578026	GK-6743/SMF 104091
Limnonectes bagoensis	MZ578027	GK-7091/SMF 106038
Limnonectes bagoensis	MZ578028	GK-7093/SMF 106039
Limnonectes bagoensis	MZ578029	GK-7142/SMF 106040
Limnonectes bagoyoma	MZ578045	GK-7110/SMF 106034
Limnonectes bagoyoma	MZ578046	GK-7111/SMF 106035
Limnonectes bagoyoma	MZ578047	GK-7136/SMF 106036
Limnonectes bagoyoma	MZ578050	HML-032
Limnonectes bannaensis	KR827879	#2004.0342
Limnonectes coffeatus	KY768796	NCSM 77785
Limnonectes coffeatus	KY768797	NCSM 77786
Limnonectes dabanus	MK688608	MVZ 258240
Limnonectes dabanus	MK688609	MVZ 258244
Limnonectes doriae	GU934330	CAS 208425
Limnonectes doriae	MG935863	USNM 587327
Limnonectes doriae	MG935864	USNM 587096
Limnonectes doriae	MG935865	CAS 248173
Limnonectes doriae	MG935866	USNM 587326
Limnonectes doriae	MG935867	USNM 587306

Table A1. *Cont.*

Species	GenBank Accession No.	Voucher
Limnonectes doriae	MG935868	USNM 587303
Limnonectes doriae	MG935871	USNM 586919
Limnonectes doriae	MG935877	USNM 586913
Limnonectes doriae	MK688573	CAS 211632
Limnonectes doriae	MK688574	CAS 229581
Limnonectes doriae	MK688575	CAS 229714
Limnonectes doriae	MK688576	FMNH 268506
Limnonectes doriae	MK688577	FMNH 268509
Limnonectes doriae	MK688578	FMNH 270111
Limnonectes doriae	MK688579	FMNH 270113
Limnonectes doriae	MW567806	ZMKU 01521
Limnonectes doriae	MW567807	ZMKU 01522
Limnonectes doriae	MW567808	ZMKU AM 01523
Limnonectes doriae	MW567809	ZMKU AM 01524
Limnonectes doriae	MW567810	ZMKU AM 01525
Limnonectes doriae	MW567811	ZMKU AM 01526
Limnonectes doriae	MW567812	ZMKU AM 01527
Limnonectes doriae	MW567813	ZMKU AM 01528
Limnonectes doriae	MW567814	ZMKU AM 01530
Limnonectes doriae	MW567815	ZMKU AM 01534
Limnonectes doriae	MW567816	ZMKU AM 01543
Limnonectes doriae	MW567817	ZMKU AM 01545
Limnonectes doriae	MW567818	ZMKU AM 01547
Limnonectes doriae	MW567819	ZMKU AM 01552
Limnonectes doriae	MZ578008	CAS 211632
Limnonectes doriae	MZ578009	CAS 211633
Limnonectes doriae	MZ578011	CAS 229581
Limnonectes doriae	MZ578012	CAS 229769
Limnonectes doriae	MZ578013	CAS 232304
Limnonectes doriae	MZ578014	CAS 245709
Limnonectes doriae	MZ578015	CAS 247238
Limnonectes doriae	MZ578016	CAS 247239
Limnonectes doriae	MZ578017	CAS 247792
Limnonectes doriae	MZ578018	CAS 247851
Limnonectes doriae	MZ578019	CAS 247908
Limnonectes doriae	MZ578020	GK-6679
Limnonectes doriae	MZ578021	GK-6682
Limnonectes doriae	MZ578022	GK-6683
Limnonectes doriae	MZ578023	GK-6684
Limnonectes doriae	MZ578024	GK-6685
Limnonectes fujianensis	AF315152	not given
Limnonectes gyldenstolpei	KR827890	#0155Y
Limnonectes gyldenstolpei	KR827891	#0175Y
Limnonectes hascheana	AY880449	MNHN 1997.5355
Limnonectes hascheanus	GU934337	FMNH 270118
Limnonectes hascheanus	GU934338	FMNH 270119
Limnonectes hascheanus	GU934349	LSUHC 6777
Limnonectes hascheanus	GU934356	CAS 229588
Limnonectes hascheanus	GU934366	THNHM 4488
Limnonectes hascheanus	GU934368	THNHM 4490
Limnonectes hascheanus	GU934369	THNHM 4491
Limnonectes hascheanus	MZ578033	CAS 247245
Limnonectes hascheanus	MZ578034	CAS 247853
Limnonectes heinrichi	AY313747	A167137
Limnonectes kohchangae	KR827893	#2003.0317
Limnonectes kohchangae	KY768809	NCSM 79546
Limnonectes lauhachindai	KP939077	ZMKU AM 01109
Limnonectes lauhachindai	MK688587	FMNH 266153
Limnonectes limborgi	AB981417	KUHE 15614

Table A1. *Cont.*

Species	GenBank Accession No.	Voucher
Limnonectes limborgi	DQ347343	isolate 1218
Limnonectes limborgi	GU934334	FMNH 271338
Limnonectes limborgi	GU934335	MVZ 258223
Limnonectes limborgi	GU934344	FMNH 262817
Limnonectes limborgi	GU934345	FMNH 262818
Limnonectes limborgi	GU934353	CAS 228513
Limnonectes limborgi	GU934354	CAS 228514
Limnonectes limborgi	GU934355	CAS 228516
Limnonectes limborgi	GU934357	CAS 229833
Limnonectes limborgi	GU934358	CAS 230378
Limnonectes limborgi	GU934359	CAS 232167
Limnonectes limborgi	GU934360	CAS 232168
Limnonectes limborgi	GU934361	CAS 232174
Limnonectes limborgi	GU934362	CAS 232189
Limnonectes limborgi	GU934363	CAS 232267
Limnonectes limborgi	GU934364	CAS 232274
Limnonectes limborgi	GU934365	CAS 232725
Limnonectes limborgi	MG935880	USNM 586920
Limnonectes limborgi	MG935881	USNM 587305
Limnonectes limborgi	MG935882	USNM 587100
Limnonectes limborgi	MZ578032	CAS 221424
Limnonectes limborgi	MZ578035	CAS 221462
Limnonectes limborgi	MZ578036	CAS 225209
Limnonectes limborgi	MZ578037	CAS 228510
Limnonectes limborgi	MZ578038	CAS 228511
Limnonectes limborgi	MZ578039	CAS 228512
Limnonectes limborgi	MZ578040	CAS 228515
Limnonectes limborgi	MZ578041	CAS 228519
Limnonectes limborgi	MZ578042	CAS 240382
Limnonectes limborgi	MZ578043	CAS 240383
Limnonectes limborgi	MZ578044	CAS 247950
Limnonectes limborgi	MZ578048	GK-7210
Limnonectes limborgi	MZ578049	GK-7216
Limnonectes limborgi	MZ578051	Kovac-3a
Limnonectes limborgi	MZ578052	Kovac-6
Limnonectes limborgi	MZ578053	PT-1597
Limnonectes limborgi	MZ578054	PT-0237
Limnonectes longchuanensis	KU599868	KIZYPX 33448
Limnonectes macrocephalus	AY313716	TNHC 61913
Limnonectes macrodon	KX055957	not given
Limnonectes macrognathus	MK688588	FMNH 268503
Limnonectes macrognathus	MK688589	FMNH 268505
Limnonectes macrognathus	MK688590	FMNH 270114
Limnonectes macrognathus	MK688591	FMNH 270115
Limnonectes plicatellus	KJ720982	LSUHC 6582
Limnonectes plicatellus	KJ720983	LSUHC 4001
Limnonectes pseudodoriae	MW567820	ZMKU AM 01553
Limnonectes pseudodoriae	MW567821	ZMKU AM 01555
Limnonectes pseudodoriae	MW567822	ZMKU AM 01563
Limnonectes pseudodoriae	MW567823	ZMKU AM 01565
Limnonectes pseudodoriae	MW567824	ZMKU AM 01567
Limnonectes pseudodoriae	MW567825	ZMKU AM 01577
Limnonectes pseudodoriae	MW567826	ZMKU AM 01579
Limnonectes pseudodoriae	MW567827	ZMKU AM 01580
Limnonectes pseudodoriae	MW567828	ZMKU AM 01582
Limnonectes pseudodoriae	MW567829	ZMKU AM 01583
Limnonectes pseudodoriae	MW567830	ZMKU AM 01585
Limnonectes savan	MK688602	NCSM 84943
Limnonectes savan	MK688603	NUOL 00061
Limnonectes taylori	KU599866	KIZ 022399

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