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A taxonomic revision of the genus *Phrynoglossus* in Indochina with the description of a new species and comments on the classification within Occidozyginae (Amphibia, Anura, Dicroglossidae)

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Abstract

We revise the frogs of the genus *Phrynoglossus* from Indochina based on data of external morphology, bioacoustics and molecular genetics. The results of this integrative study provide evidence for the recognition of three distinct species, one of which we describe as new. *Phrynoglossus martensii* has a vast geographic distribution from central and southern Thailand across southern China to Vietnam, Laos, and Cambodia. *Phrynoglossus myanhessei* **sp. nov.** is distributed in central Myanmar whereas *Phrynoglossus magnapustulosus* is restricted to the Khorat Plateau, Thailand. These three species occur in allopatry and differ in their mating calls, external morphology, and in genetic distances of the 16S gene of 3.8–5.9%. Finally, we discuss and provide evolutionary evidence for the recognition of *Phrynoglossus* as a genus distinct from *Occidozyga*. Members of both genera form reciprocal monophyletic groups in our analyses of mtDNA data (16S) and are well differentiated from each other in morphology and ecology. Furthermore, they differ in the amplexus mode with *Phrynoglossus* having an inguinal amplexus whereas it is axillary in *Occidozyga*. We further provide a *de novo* draft genome of the holotype based on short-read sequencing technology to a coverage of 25-fold. This resource will permanently link the genetic characterization of the species to the name-bearing type specimen.

Key words

amplexus mode, bioacoustics, cryptic species diversity; Dicroglossidae, genome; Khorat Plateau, Myanmar, new species, *Occidozyga*; *Phrynoglossus myanhessei* sp. n., Thailand

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Introduction

Southeast Asia is recognized as a major global biodiversity hotspot (Myers et al. 2000; Mittermeier 2004; Corlett 2014), and the amphibian order Anura is well represented in this region. However, it seems that the actual diversity of anurans in this part of the world is still grossly underestimated due to poor sampling of areas with difficult or dangerous access and because most of the supposedly wide-spread species actually constitute species complexes of two or more distinct species (e.g., Funk et al. 2011; Hasan et al. 2012; Köhler et al. 2019). During field work in Myanmar and Thailand we came across variation in the advertisement call of the common mud or puddle frogs, in this region all currently referred to as *Occidozyga martensii* Peters, 1867 (Niyomwan et al. 2019; Frost 2020). These observations prompted us to collect this group of frogs to obtain a reasonable geographic coverage with the goal to evaluate whether these really are a single species occurring in a large geographic area or if these actually represent a complex of several – morphologically similar (cryptic) – species with smaller geographic ranges.

The generic placement of *martensii* in either *Phrynoglossus* Peters, 1867 or *Occidozyga* Kuhl and van Hasselt, 1822 has caused some controversy among recent authors. It seems that the majority of authors have treated *Phrynoglossus* as a synonym of *Occidozyga* (e.g., Duellman and Trueb 1994; Nguyen et al. 2009; Frost 2020) whereas others have recognized them as two valid genera (e.g., Manthey and Grossmann 1997; Ohler and Dubois 1999; Ziegler, 2002; Köhler et al. 2018). Here we provide a short summary of the taxonomic history of *Phrynoglossus* and *Occidozyga*:

The genus name *Occidozyga* was introduced by Kuhl and van Hasselt 1822. Its type species (by subsequent designation of Stejneger 1925) was described as *Rana lima* Gravenhorst 1829 a few years later. In 1859, Günther described *Oxyglossus laevis* based on material from the Philippines. *Oxyglossus* Tschudi 1838 is an objective synonym of *Occidozyga* Kuhl and van Hasselt 1822 according to Ohler and Dubois (1999) whereas *Ooeidozyga*, *Oxydozyga*, *Occidogyna* are incorrect subsequent spellings of *Occidozyga* (Dubois 1981, Frost 2020). The genus name *Oreobatrachus* Boulenger 1896 is a synonym of *Phrynoglossus* Peters 1867 according to Smith (1931).

In 1896, Boulenger introduced his new species *Oreobatrachus baluensis* from „Mount Kina Balu, North Borneo [= Sabah]“, Malaysia (Borneo). Smith (1931) placed *Oreobatrachus* Boulenger 1896 in the synonymy of *Phrynoglossus* Peters 1867. With the introduction of the latter genus, Peters (1867) described the new species *P. martensii*, the holoype of which he had received from Bangkok, Thailand. In 1877, Peters described *Microdiscopus sumatranus* from Sumatra, Indonesia. Smith (1916) discussed the taxa *laevis*, *lima* and *martensii* and considered *martensii* to be a subspecies of *laevis*. Also, he described and illustrated the tadpoles of *lima* and *martensii* and provided natural history notes for the two taxa. He stated (Smith 1916: 175) that “*O. lima* is strictly aquatic in its habits” whereas *martensii*, “although never far from water, is seldom to be found in it.” Taylor (1922) introduced the new species *Micrixalus diminutiva* based on specimens from “near Pasananka, Zamboanga, Mindanao”, Philippines. Inger (1954) transferred the latter taxon to the genus *Ooeidozyga*. In 1927, Smith described two new species from Sulawesi, Indonesia: *Ooeidozyga semipalmata* from „Lowah, near Mt. Bonthain“, and *Ooeidozyga celebensis* from “Djikoro, Mt. Bonthain”. Based on specimens from “Rana Mese, 1200 m. H., West-Flores”, Indonesia, Mertens (1927) described *Oxydozyga floresiana*. In 1958, Taylor and Elbel introduced their new species *Occidozyga magnapustulosa* with type locality in “Ban Na Phua (subvillage), Kan Luang (village), Na Kae (district), Nakhon Phanom (province), Thailand, elevation approx. 200 m”. More recently, Iskandar et al. (2011) analyzed the morphological variation in populations of frogs related to *Occidozyga semipalmata* and recognized the populations from „Bantayan, Mount Tompotika, Balantak Mountains …, Desa (=Village) Bualemo, … Sulawesi Province, Indonesia“ as a distinct species that they named *Occidozyga tompotika*.

In his treatment of the herpetofauna of Mount Kinabalu, Borneo, Smith (1931) pointed out the unique tongue morphology of *Ooeidozyga lima*, strikingly different from the other species of that genus, and proposed to retain only *O. lima* in the genus and to transfer the remaining species to the genus *Phrynoglossus*. However, this proposal was not widely accepted by subsequent authors (e.g., Inger 1954, 1996) who preferred to maintain all species in a single genus (i.e., *Occidozyga* respectively its synonym *Ooeidozyga*). Inger (1996) argued that of the five supposedly diagnostic characters listed for *Phrynoglossus*, only one, the tongue morphology, would distinguish the species of the latter genus from *Occidozyga*.

Further evidence for considering *Phrynoglossus* as a synonym of *Occidozyga* was provided in the large-scale molecular phylogeny of amphibians of Pyron and Wiens (2011) where the two species of *Phrynoglossus* were nested within the species of *Occidozyga*, thereby rendering the latter genus paraphyletic if *Phrynoglossus* was recognized as a valid genus. Basal to the clade containing the species of *Phrynoglossus* and *Occidozyga* is *Ingerana* in the tree of these authors. However, aside from tongue morphology, there is additional evidence in favor of recognizing these two groups as distinct genera (see our Results section), and we therefore recognize *Phrynoglossus* as a valid genus distinct from *Occidozyga*.

Materials and methods

Specimens examined for this study were personally collected by GK, NLT, and PT (see Appendix 1 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 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. 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 Köhler (2012).

In evaluating species' boundaries within and among populations, we followed the evolutionary species concept (Simpson 1951; Wiley 1978). 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); NED (nostril–eye distance); HNL (hand length); SHL (shank length); SL (snout length); SVL (snout–vent length); TED (tympanum–eye distance); THL (thigh length); TYD (longitudinal tympanum diameter). Webbing formulae follow (Savage and Heyer 1997). Terminology of snout shape follows Heyer et al. (1990).

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 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 141 Hz; grid spacing of 21.5 Hz; overlap 90%. Frequency information was generated through Fast Fourier Transformation (FFT, width 2,048 samples). Temporal measurements of calls such as repetition rates, duration of notes, and number of pulses, were measured on the waveforms. Terminology in call descriptions follows Köhler et al. (2017). The map was created using ArcMap 10.4. Additionally to the specimens examined by us, we also plotted specimens from the Field Museum (FMNH), Chicago, not examined by us.

Marker based analysis

We extracted DNA following the protocol of Ivanova et al. (2006). To eliminate potential PCR-inhibiting contaminants, the tissue samples were incubated for 14 hours 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 done at SMF for the samples from Myanmar and at Chulalongkorn University for the samples from Thailand. We generated 35 new sequences for this study (see Appendix 2). Additionally, we downloaded relevant 16S sequences from GenBank (Appendix 2). Because the frogs of the genus *Ingerana* Dubois, 1987 are supposedly the closest relatives of *Phrynoglossus* and *Occidozyga* (see Pyron and Wiens 2011) we included sequences of *Ingerana tenasserimensis* (Sclater 1892) in our analyses. Our dataset contains the type species of *Ingerana* (i.e., *Rana tenasserimensis* Sclater, 1892), *Occidozyga* (i.e., *Rana lima* Gravenhorst, 1829), and *Phrynoglossus* (i.e., *Phrynoglossus martensii* Peters, 1867).

We aligned the sequences with MUSCLE (Edgar 2004) using the default settings in Geneious 6.1.2. (Kearse et al. 2012). For software applications, sequence data formatting was converted using the online server Alter (Glez-Peña et al. 2010). The best substitution model for each gene was identified using PartitionFinder2 (Lanfear et al. 2017), with linked branch lengths via PhyML 3.0 (Guindon et al. 2010). Model selection used the corrected (for finite sample size) Akaike Information Criterion (AICc) (Burnham and Anderson 2002). *Limnonectes limborgi* (GK-7110) was used as outgroup (Pyron and Wiens 2011).

Bayesian Inference analyses (BI) used MrBayes 3.2 (Ronquist et al. 2012) 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 PRSF (Potential Scale Reduction Factor), which should approach 1.0 as runs converge (Gelman and Rubin 1992). We used the IQTree webserver (Trifinopoulos et al. 2016) 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 (Minh et al. 2013), plus 10,000 replicates of Shimodaira-Hasegawa approximate likelihood ratio (SH-aLRT), which proved to be accurate with a high statistical power (Guindon et al. 2010). We used FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree) for viewing the trees. We estimated evolutionary genetic divergence, computing uncorrected pairwise distances with MEGA 7.0.26 (Kumar et al. 2016) 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 (Ogilvie et al. 2017; Bouckaert et al. 2018) with 1,000,000 generations for the MCMC model. The resulting tree was visualized using DensiTree 2.2.6 (Bouckaert and Heled 2014). Automatic barcode gap discovery (ABGD) (Puillandre et al. 2012) was run through its webserver (http://www.abi.snv.jussieu.fr/public/abgd/abgdweb.html), with using Simple Distance and default values for Prior Intraspecific divergence, except for relative gap width (1.5), which did not work for our genes (see also Kekkonen et al. 2015). Because high values in relative gap-width tend to overly split species (Yang et al. 2016), we used an intermediate value of 1.0.

Whole genome sequencing and assembly

De novo whole genome sequencing and assembly began with genomic DNA extraction from muscle tissue of the holotype according to standard phenol/chloroform procedures (Sambrook and Russell 2001). The resulting DNA was resuspended in TE buffer (10mM Tris-Cl, 0.1 mM EDTA) and stored at –20°C. Quality checks for high molecular weight DNA were performed by agarose gel electrophoresis (Sambrook and Russell 2001). The DNA sample was shipped on dry ice to Novogene (UK) for short read Illumina genome sequencing. One genomic library (insert size: 350 bp) was prepared and 150 bp paired-end reads were sequenced on an Illumina NovaSeq 6000 platform (San Diego, CA). Raw reads are deposited under the accession number SRR13288470.

A *k*-mer profile was generated from the raw reads using Jellyfish 2.3.0 (Marçais and Kingsford 2011) and analyzed in the GenomeScope webserver (Vurture et al. 2017). Raw reads were trimmed for low quality regions and adapter sequences and filtered for possible contamination using Trimmomatic 0.39 (Bolger et al. 2014) and Kraken 2.0.9 (Wood et al. 2019) respectively. Best *k*-mer length was estimated by KmerGenie 1.7051 (Chikhi and Medvedev 2014). Nuclear genome assembly was conducted in Velvet 1.2.10 (Zerbino and Birney 2008) without reporting contigs smaller than 500 bp. The mitochondrial genome was assembled from the genomic data separately using NOVOplasty 4.2 (Dierckxsens et al. 2017). Annotation of the mitochondrial genome was manually merged and curated in Geneious Prime 2020.2.3 (https://www.geneious.com) from automatic annotations of GeSeq (Tillich et al. 2017) and MITOS2 (Donath et al. 2019) webservers. Scaffolds matching the mitochondrial genome assembly and scaffolds smaller than 500 bp were removed from the nuclear genome assembly. Finally, the mitochondrial genome assembly was added and quality was checked by backmapping the reads to the assembly with bwa mem 0.7.17-r1188 (Li 2013), searching for possible contamination with Blobtools 1.1.1 (Laetsch and Blaxter 2017) and screening for single-copy orthologs with BUSCO 4.1.4 (Simão et al. 2015). For detailed description see Appendix 3.

Results and Conclusions

The final 16S alignment for 69 samples (genera *Ingerana*, *Occidozyga*, and *Phrynoglossus*) was 599 nucleotide positions long. GTR+G was determined to be the best fitting model of sequence evolution. The trees obtained by BI, ML, *BEAST, and ABGD analyses show a high degree of congruence at well-supported nodes, with some differences in branch arrangement at poorly supported nodes (Figs. 1, 2 and 3). Our final phylogenetic analyses recover the deep divergences between the genera *Ingerana*, *Occidozyga*, and *Phrynoglossus* (Figs. 1, 2 and 3). These three genera form monophyletic groups in all analyses.

Ingerana is the sister clade to a clade containing *Occidozyga* plus *Phrynoglossus*. There is much genetic structure in the *Occidozyga* clade indicating the possible presence of cryptic species among the populations currently referred to as *O. lima* (Gravenhorst, 1829). Among the specimens here referred to the genus *Phrynoglossus*, four major clades are recognizable and also supported by the ABGD analysis (Fig. 3). Clade 1 (= Group 7 in our ABGD analysis) contains specimens from southern Thailand including Bangkok (= type locality of *P. martensii*) and are therefore considered to represent the "true" *P. martensii*. Clade 2 (= Group 8 in our ABGD analysis) contains specimens from northern Thailand, southern China, Vietnam, Laos, and Cambodia. Clade 3 (= Group 11 in our ABGD analysis) is geographically restricted to the Khorat Plateau, Thailand, and includes specimens from Ban Kan Luang (= type locality of *P. magnapustulosus* Taylor and Elbel, 1958) and are therefore considered to represent the latter species. Finally, Clade 4 (= Group 10 in our ABGD analysis) contains specimens from central and lower Myanmar. We consider these four clades as candidate species. The genetic distances among these clades vary from 3.5% to 5.9% (Table 1).

In bioacoustics, the advertisement calls of males from Clades 1, 3, and 4 can be readily distinguished whereas the calls of males from Clades 1 and 2 are exceedingly similar (Table 2; Fig. 4). Males of *P. magnapustulosus* (our Clade 3) emit an advertisement call that sounded "mourning" to our ears ("maaaaaaa"). It has a duration of 243–437 ms (mostly in the 300 ms range) with its dominant frequency mostly in the range of 3500–3700 Hz (3187–3790 Hz) and a gap duration between calls of 2.3–5.3 s, mostly 3–4 s. On the contrary, the call of *P. martensii* (Clade 1) is very short (sounding like "bic") with a duration of 40–48

Figure 1. Phylogenetic tree of frogs of the subfamily Occidozyginae from a Bayesian Inference analysis of the mitochondrial marker 16S. A scale bar of genetic distance is indicated. Numbers at nodes are bootstrap values (left) and Bayesian posterior probabilities (right), but only for nodes with bootstrap values higher than 75. The tree is rooted using *Limnonectes limborgi*. — Abbreviations: *P.* = *Phrynoglossus*; *myanh.* = *myanhessei* **sp. n.**; *magn.* = *magnapustulosus*; *I. ten.* = *Ingerana tenasserimensis*.

ms and a dominant frequency of 3467–3596 Hz. The gap duration between calls varies from 2.8–3.9 s. The calls of males from Myanmar (Clade 4) are somewhat similar to the calls of *P. martensii* but are of longer duration (82–114 ms) and lower pitched (dominant frequency 2454–2885 Hz). The gap duration between calls varies from 1.8–4.8 s. The advertisement call of our "cf. *martensii*" (Clade 2) is very similar to the call of *P. martensii* (Clade 1) and differs only in having mostly a shorter gap duration between calls (0.5–3.0 s, mostly < 2.5 s in "cf. *martensii*" versus 2.8–3.9 in *P. martensii*).

In external morphology, specimens of these four clades are somewhat conservative in external morphology but show differences in some morphometric values (Fig. 5;

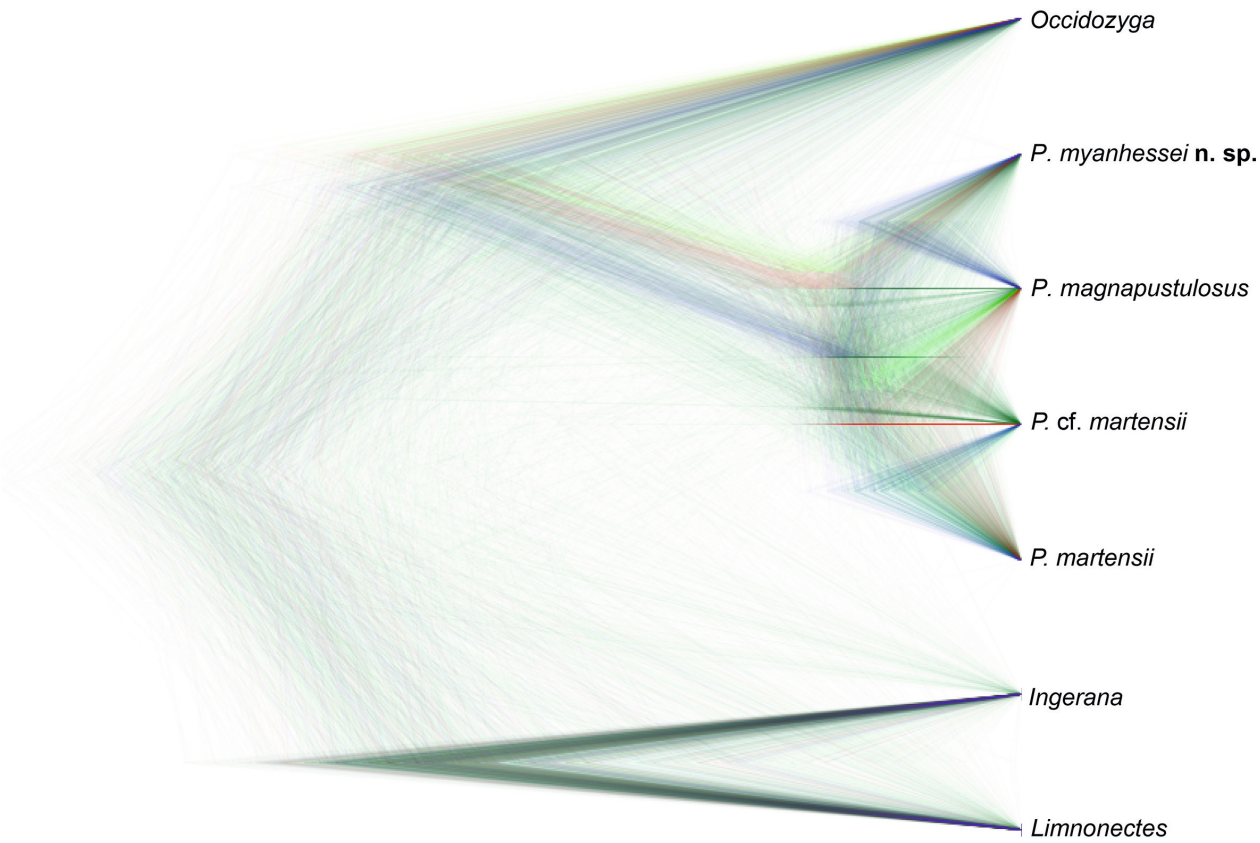

Figure 2. Species tree inferred with *BEAST showing density of trees proportional to frequency of occurrence drawn in DensiTree. Abbreviations: *P.* = *Phrynoglossus.*

Table 3) as well as in the amount of toe webbing and in some details of coloration (see respective diagnosis sections below). Also, there is sexual dimorphism evident in several morphometric characters such as SVL, SHL, FL, and HL. *Phrynoglossus magnapustulosus* have the smallest SVL (males < 20 mm versus > 20 mm in the other clades) and also have a much larger ratio TYD/SVL compared to those from the other clades.

Thus, based on the results of the analyses of mtDNA data, morphology, and bioacoustics, we recognize three of the four clades as defined above as species level units (i.e., our OTUs 1, 3 and 4) whereas we tentatively consider our OTU 2 to be conspecific with our OTU 1 since we are unable to find any diagnostic differences in morphology or bioacoustics that would separate these two OTUs. Based on the respective type localities, our Clade 1 is assigned to *Phrynoglossus martensii* whereas our Clade 3 is referred to as *P. magnapustulosus*. Clade 4 represents an undescribed species for which no name is available. Thus, we describe the *Phrynoglossus* from central and lower Myanmar as a new species below.

Regarding the recognition of *Phrynoglossus* as a valid genus distinct from *Occidozyga*, we find support by the results of our genetic and morphologic analyses as well as our field observations on the amplexus mode of these frogs. *Phrynoglossus* has an inguinal amplexus whereas *Occidozyga* has an axillary amplexus (Fig. 6). The two genera differ readily in tongue morphology with *Occidozyga* having a very slender, worm-like tongue whereas the tongue is fleshy and thick in *Phrynoglossus* (Fig. 7a,b) Also, species of the two groups differ strikingly in skin texture (Fig. 7c,d) and mucosome, at least judged from touching these frogs with bare hands: species of *Phrynoglossus* have a smooth skin and are extremely slimy and thus difficult to constrain manually. On the other hand, *Occidozyga* has a rough skin and is not slimy at all. Finally, the two groups differ in ecology: species of *Phrynoglossus* are terrestrial frogs that are usually found on mud at the shore of small water bodies whereas *Occidozyga* is a fully aquatic species that always remains in the water body. For the issue of possible paraphyly of this group of frogs see also our results of the analysis of the mtDNA data used in this study.

Thus, we formally resurrect *Phrynoglossus* from the synonymy of *Occidozyga* and define the two genera as follows (data also from Ohler and Dubois 1999):

Occidozyga Kuhl & van Hasselt, 1822

Type species. *Rana lima* Gravenhorst, 1829.

Diagnosis. A genus of Asian frogs of the subfamily Occidozyginae Fei, Ye, and Huang, 1990 of the family Dicroglossidae Anderson, 1871, that differs from all other genera of its subfamily by having the following combination of characters: (1); tongue slender, worm-like; (2) vomerine teeth absent; (3) tips of fingers and toes pointed; (4) tympanum hidden; (5) skin not covered by extensive mucous, feels dry to touch in life frogs; (6) throat lining

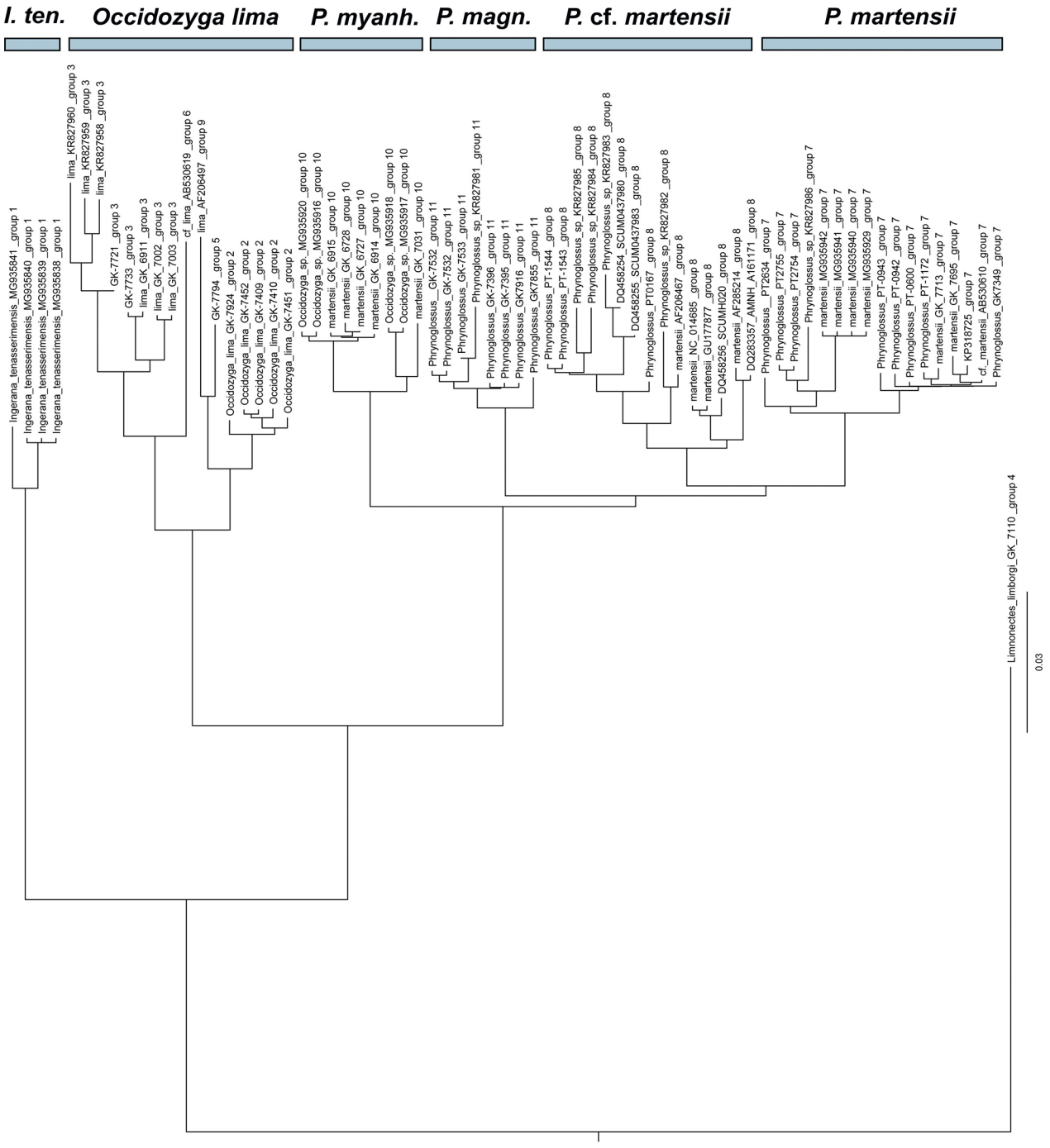

Figure 3. Species delimitation analysis of frogs of the subfamily Occidozyginae using the Automatic Barcode Gap Discovery (ABGD) approach; initial partition with prior maximal distance at 0.00167. See text for details. Abbreviations as in Fig. 1.

whitish with longitudinal brown stripe; (7) life style fully aquatic; (8) amplexus axillary.

Content. *Occidozyga lima* (Gravenhorst, 1829)

Phrynoglossus Peters, 1867

Type species. *Phrynoglossus martensii* Peters, 1867

Diagnosis. A genus of Asian frogs of the subfamily Occidozyginae Fei, Ye, and Huang, 1990 of the family Dicroglossidae Anderson, 1871, that differs from all other genera of its subfamily by having the following combination of characters: (1); tongue fleshy, swollen; (2) vomerine teeth absent; (3) tips of fingers and toes slightly swollen; (4) tympanum moderately distinct; (5) skin covered by extensive mucous, feels slimy to touch in life frogs; (6) throat lining uniformly grey; (7) life style semiaquatic; (8) amplexus inguinal.

Content. *Phrynoglossus baluensis* (Boulenger, 1896), *Phrynoglossus celebensis* (Smith, 1927), *Phrynoglossus diminutivus* (Taylor, 1922), *Phrynoglossus floresianus* (Mertens, 1927), *Phrynoglossus laevis* (Günther, 1859), *Phrynoglossus magnapustulosus* (Taylor and Elbel,

Table 1. Uncorrected pairwise distances for the Occidozyginae plus *Limnonectes* (outgroup) included in this study. For details see text.

	Ingerana	**Occidozyga**	**Limnonectes**	**martensii**	**cf._martensii**	**„Myanmar“**
Occidozyga	0.189					
Limnonectes	0.192	0.193				
martensii	0.198	0.132	0.203			
cf._martensii	0.202	0.125	0.213	0.035		
„Myanmar“	0.203	0.139	0.209	0.054	0.051	
magnapustulosus	0.208	0.127	0.207	0.045	0.038	0.059

Table 2. Selected bioacoustic parameters of the species related to *Phrynoglossus martensii*. 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)	**Freq 5%** (Hz)	**Freq 95%** (Hz)
GK-7475_0544 (n=23 calls) Roi Et Prov., Thailand	*magnapustulosus*	0.243–0.354 (0.291±0.024)	3187–3682 (3447±175)	2.25–4.28 (3.15±0.58)	1680–1938 (1790±74)	4199–5383 (4823±388)
GK-7465_0543 (n=15 calls) Roi Et Prov., Thailand	*magnapustulosus*	0.322–0.437 (0.387±0.031)	3725–3790 (3761±20)	2.67–5.31 (3.92±0.67)	1744–2131 (1894±94)	4091–4177 (4150±29)
GK-7476_0546 (n=9 calls) Roi Et Prov., Thailand	*magnapustulosus*	0.262–0.287 (0.277±0.008)	3750–3750 (3750±0)	2.53–3.20 (2.77±0.19)	1640–1734 (1708±32)	4406–4453 (4427±23)
GK-6727_0183 (n=7 calls) EYU, Myanmar	n. sp. “Myanmar”	0.085–0.114 (0.104±0.009)	2454–2476 (2467±11)	2.34–2.90 (2.66±0.23)	1357–1809 (1529±156)	2820–2863 (2839±18)
GK-6926_0008 (n=21 calls) EYU, Myanmar	n. sp. “Myanmar”	0.079–0.109 (0.098±0.008)	2756–2885 (2839±41)	2.47–4.78 (3.31±0.67)	1766–2347 (2119±168)	3187–3316 (3265±37)
Not collected (n=26 calls) Thanlyin, Myanmar	n. sp. “Myanmar”	0.082–0.102 (0.090±0.005)	2541–2584 (2566±18)	1.84–2.80 (2.18±0.32)	1357–2110 (1831±238)	2929–3015 (2975±26)
Goutte_9357 (n=24 calls) Taiwan	cf. *martensii*	0.041–0.051 (0.046±0.003)	3876–3941 (3914±17)	2.12–2.99 (2.39±0.32)	2304–3488 (3027±414)	4264–4737 (4493±128)
Goutte_9358 (n=28 calls) Taiwan	cf. *martensii*	0.038–0.052 (0.043±0.003)	3919–3962 (3934±15)	1.94–2.78 (2.37±0.65)	2864–3553 (3356±414)	4264–4371 (4300±24)
Ziegler TZ-473 (n=24 calls) Vietnam	cf. *martensii*	0.032–0.046 (0.037±0.004)	3338–3553 (3444±70)	0.53–0.90 (0.72±0.09)	1873–3144 (2874±275)	3725–4027 (3830±76)
Chiang Mai (n=10 calls) Thailand	cf. *martensii*	0.033–0.043 (0.037±0.003)	3660–3704 (3689±14)	1.91–2.56 (2.19±0.20)	2670–3165 (2922±130)	4134–4435 (4254±111)
Bangkok (n=14 calls) Thailand	*martensii*	0.040–0.048 (0.043±0.002)	3467–3596 (3544±37)	2.80–3.86 (3.42±0.34)	2261–3165 (2689±257)	3919–4155 (4028±69)

Table 3. Selected measurements and proportions of the species of *Phrynoglossus*. Range is followed by mean value and standard deviation in parentheses. For abbreviations see text.

		P. martensii ♂ 7 | ♀ 8	***P. magnapustulosus*** ♂ 8 | ♀ 12	***P. myanhessei*** ♂ 9 | ♀ 6
SVL	males	19.77–23.31 (21.42±1.28)	17.21–19.68 (17.91±0.804)	22.00–27.35 (24.06±1.65)
	females	25.55–30.35 (27.60±2.061)	16.90–24.30 (21.66±2.26)	28.38–31.27 (29.67±1.15)
SHL / SVL	males	0.441–0.509 (0.479±0.021)	0.442–0.557 (0.509±0.036)	0.457–0.571 (0.506±0.032)
	females	0.436–0.479 (0.462±0.016)	0.402–0.548 (0.478±0.041)	0.466–0.592 (0.500±0.047)
FL / SVL	males	0.471–0.535 (0.491±0.024)	0.473–0.564 (0.527±0.031)	0.433–0.535 (0.476±0.037)
	females	0.432–0.488 (0.468±0.021)	0.439–0.567 (0.482±0.045)	0.407–0.491 (0.449±0.033)
HL / SVL	males	0.341–0.383 (0.359±0.015)	0.313–0.377 (0.341±0.024)	0.268–0.378 (0.330±0.031)
	females	0.311–0.383 (0.344±0.022)	0.272–0.347 (0.304±0.028)	0.272–0.304 (0.289±0.011)
HW / SVL	males	0.336–0.401 (0.357±0.023)	0.335–0.394 (0.364±0.021)	0.286–0.369 (0.338±0.029)
	females	0.338–0.388 (0.351±0.017)	0.295–0.360 (0.336±0.019)	0.283–0.354 (0.312±0.025)
HL / HW	males	0.909–1.077 (1.011±0.063)	0.855–1.030 (0.937±0.058)	0.897–1.191 (0.980±0.092)
	females	0.920–1.020 (0.980±0.031)	0.779–1.057 (0.907±0.098)	0.808–1.043 (0.929±0.093)
IOD / SVL	males	0.071–0.091 (0.086±0.007)	0.093–0.135 (0.112±0.015)	0.090–0.132 (0.107±0.013)
	females	0.073–0.084 (0.077±0.003)	0.084–0.137 (0.106±0.018)	0.077–0.163 (0.104±0.032)

Table 3 continued.

		P. martensii ♂ 7 | ♀ 8	*P. magnapustulosus* ♂ 8 | ♀ 12	*P. myanhessei* ♂ 9 | ♀ 6
TYD / SVL	males	0.042–0.068 (0.052±0.010)	0.055–0.099 (0.082±0.014)	0.029–0.066 (0.050±0.012)
	females	0.035–0.052 (0.043±0.006)	0.062–0.102 (0.080±0.012)	0.034–0.064 (0.051±0.010)
EYD / SVL	males	0.109–0.138 (0.122±0.010)	0.091–0.143 (0.116±0.017)	0.094–0.140 (0.112±0.015)
	females	0.095–0.119 (0.104±0.008)	0.082–0.118 (0.100±0.012)	0.077–0.132 (0.096±0.019)
NED / SVL	males	0.056–0.071 (0.064±0.006)	0.064–0.123 (0.086±0.018)	0.061–0.101 (0.083±0.016)
	females	0.055–0.066 (0.060±0.004)	0.058–0.086 (0.074±0.009)	0.060–0.145 (0.090±0.033)
TYD / EYD	males	0.357–0.512 (0.425±0.063)	0.482–0.920 (0.722±0.147)	0.291–0.653 (0.460±0.146)
	females	0.328–0.515 (0.411±0.060)	0.591–0.962 (0.807±0.120)	0.385–0.727 (0.550±0.143)

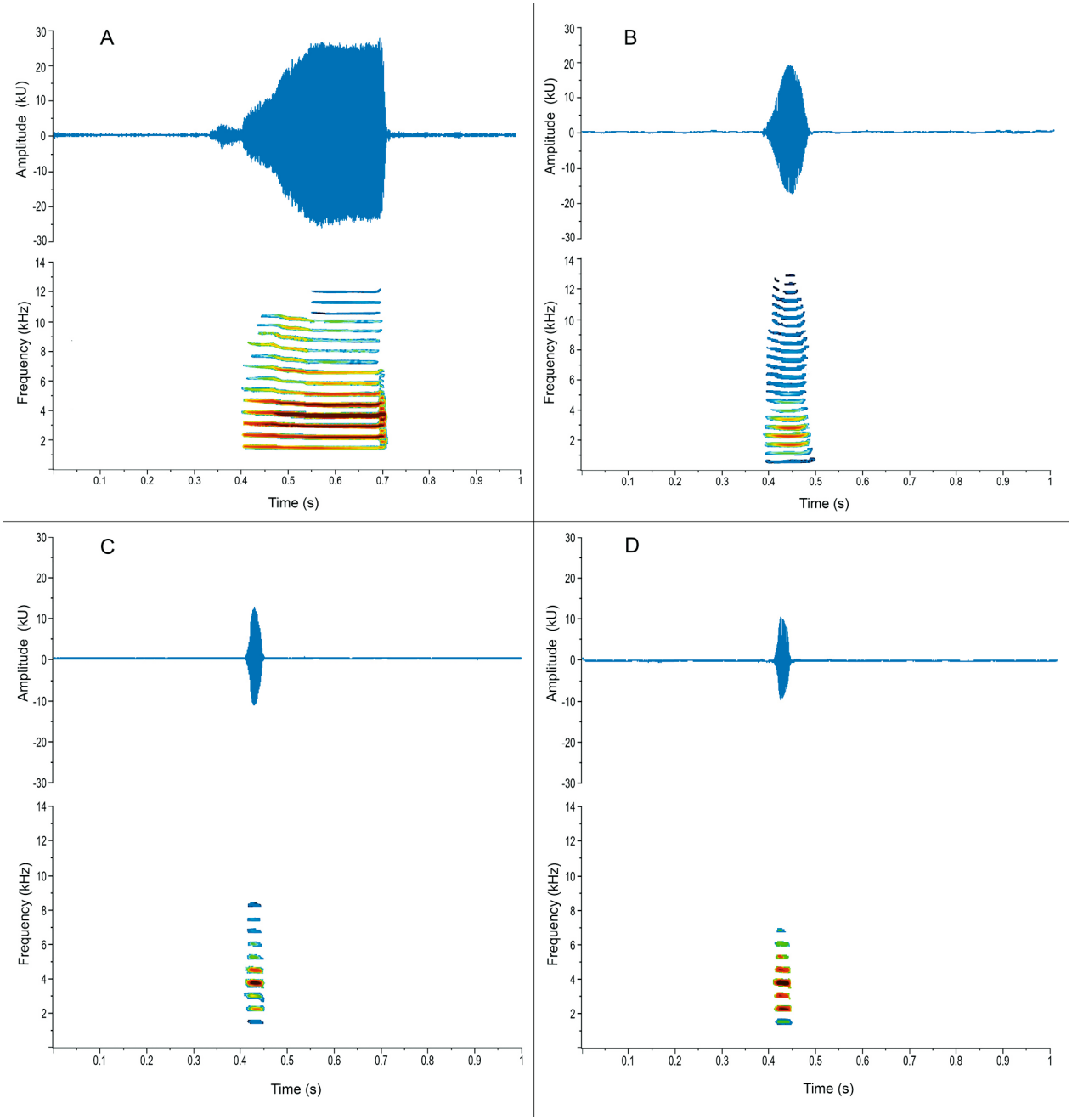

Figure 4. Advertisement calls of male *Phrynoglossus*. (A) *P. magnapustulosus*, GK-7395; (B) *P. myanhessei* **n. sp.**, SMF 103799; (C) *P. martensii,* PT-2634 (Bangkok); (D) *P. martensii,* PT-2076 (Chiang Mai).

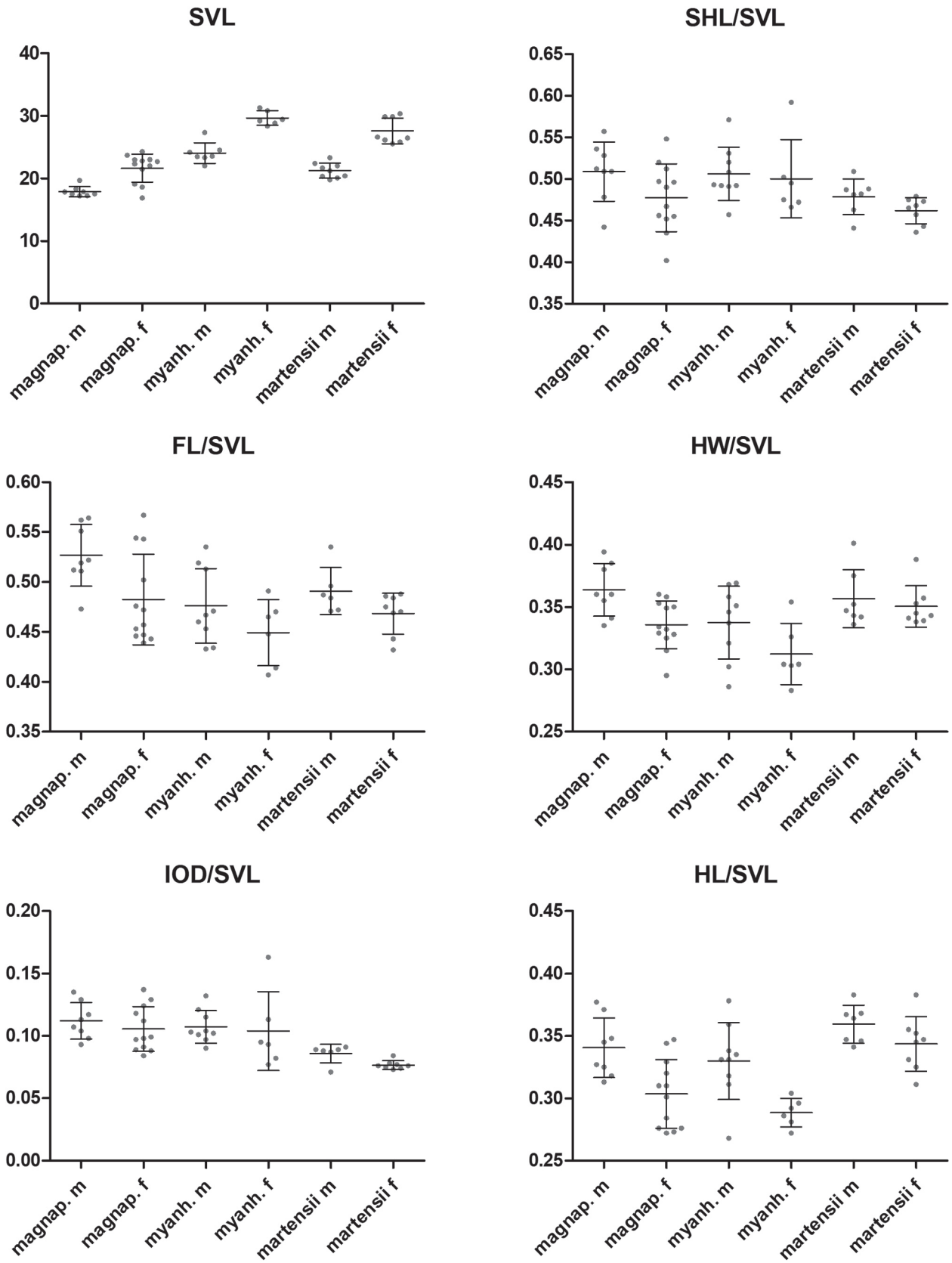

Figure 5 – part 1. Scatter plots illustrating morphological variation in the species of *Phrynoglossus*. magnap. = *P. magnapustulosus*; m = male; f = female. For abbreviations of morphological characters see text. Abbreviations of taxon names as in Fig. 1.

1958), *Phrynoglossus martensii* Peters, 1867, *Phrynoglossus semipalmatus* (Smith, 1927), *Phrynoglossus sumatranus* (Peters, 1877), *Phrynoglossus tompotika* (Iskandar, Arifin, and Rachmanasah, 2011).

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

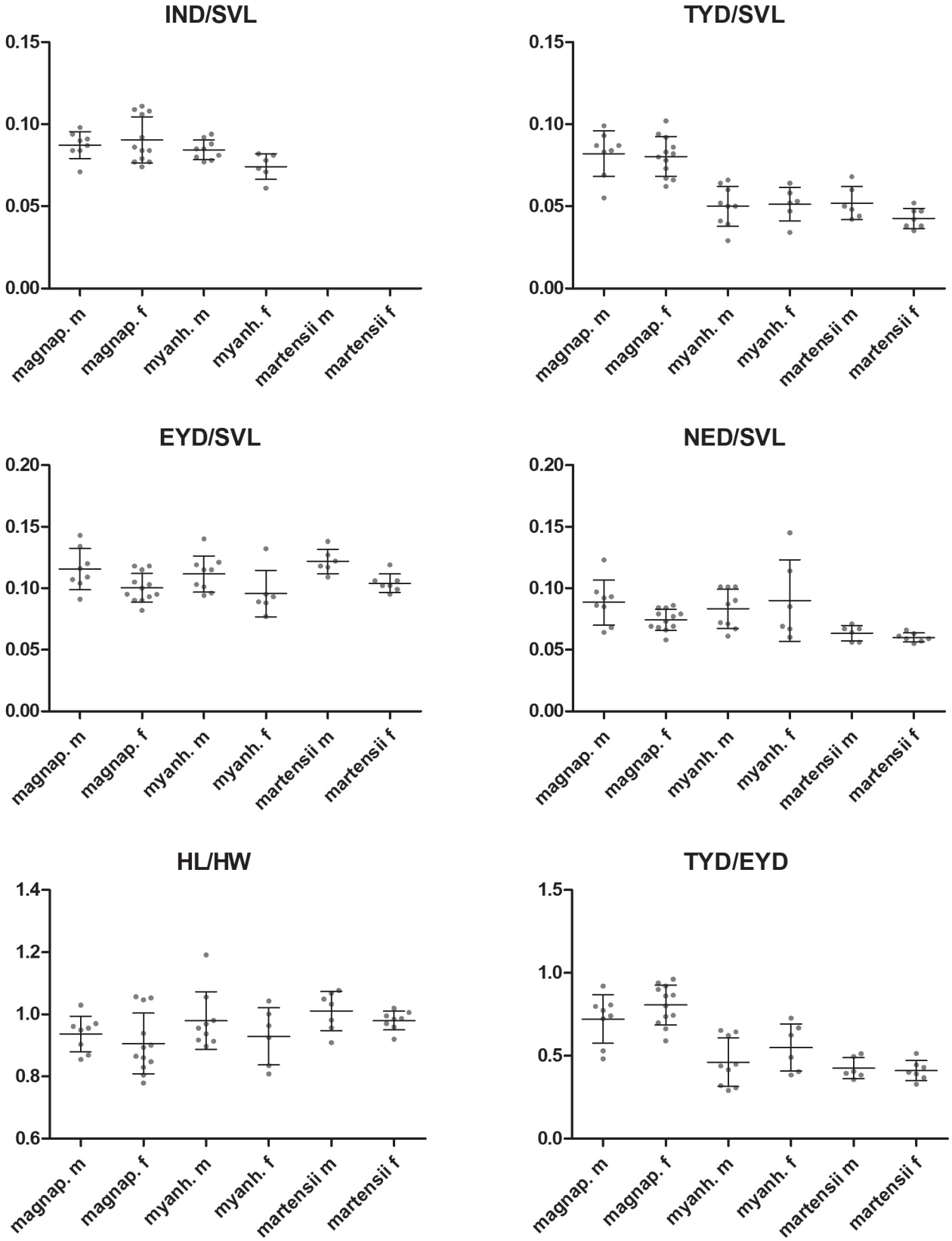

Figure 5 – part 2. Scatter plots illustrating morphological variation in the species of *Phrynoglossus*. magnap. = *P. magnapustulosus*; m = male; f = female. For abbreviations of morphological characters see text. Abbreviations of taxon names as in Fig. 1.

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:A978D105-6D7F-4858-BA27-8639B76A6039. The LSID registration and any associated information can be viewed in a web browser by adding the LSID to the prefix "http://zoobank.org."

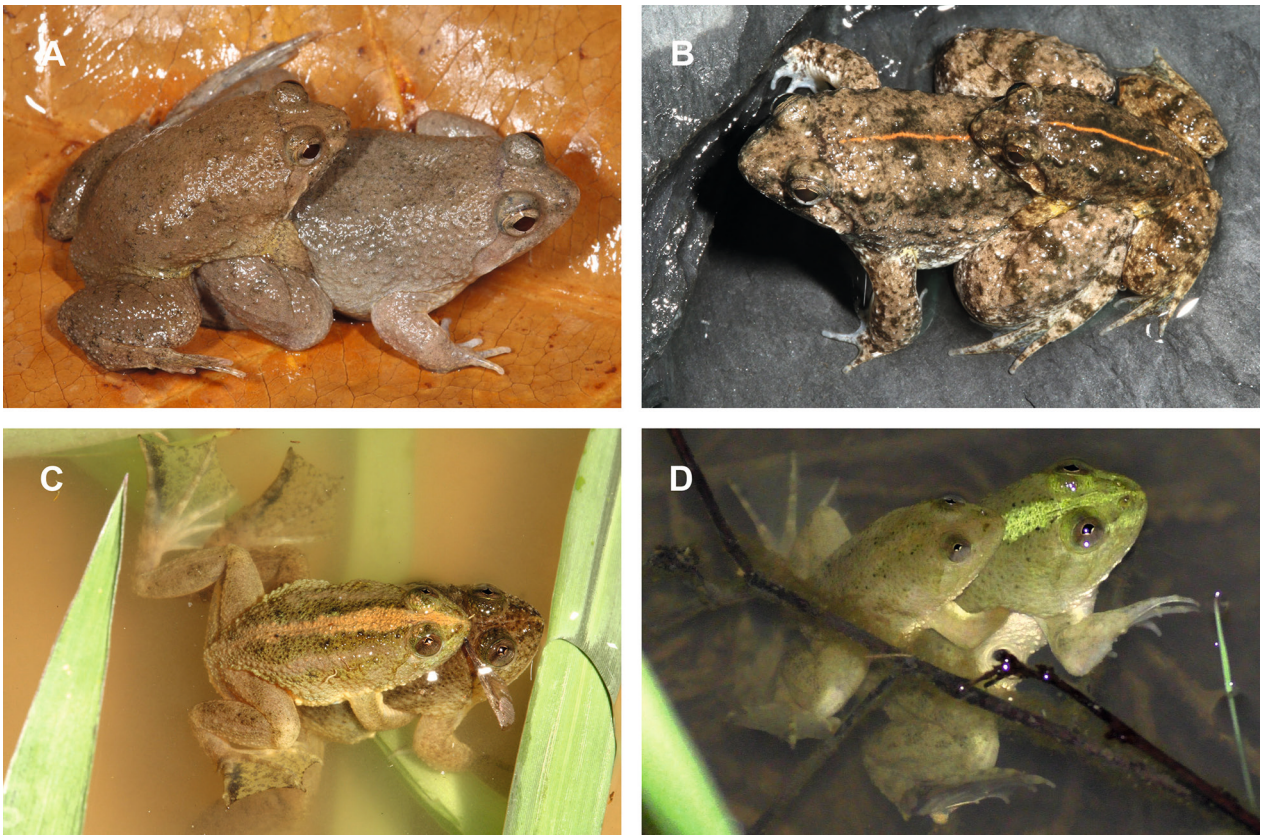

Figure 6. Frogs of *Phrynoglossus* and *Occidozyga* in amplexus. (A) *P. myanhessei* **n. sp.**, SMF 103797–98; (B) *P. magnapustulosus*, not collected; (C) *O. lima,* not collected (Magwe State, Myanmar); (D) *O. lima,* not collected (Roi Et province, Thailand). Photos by G.K.

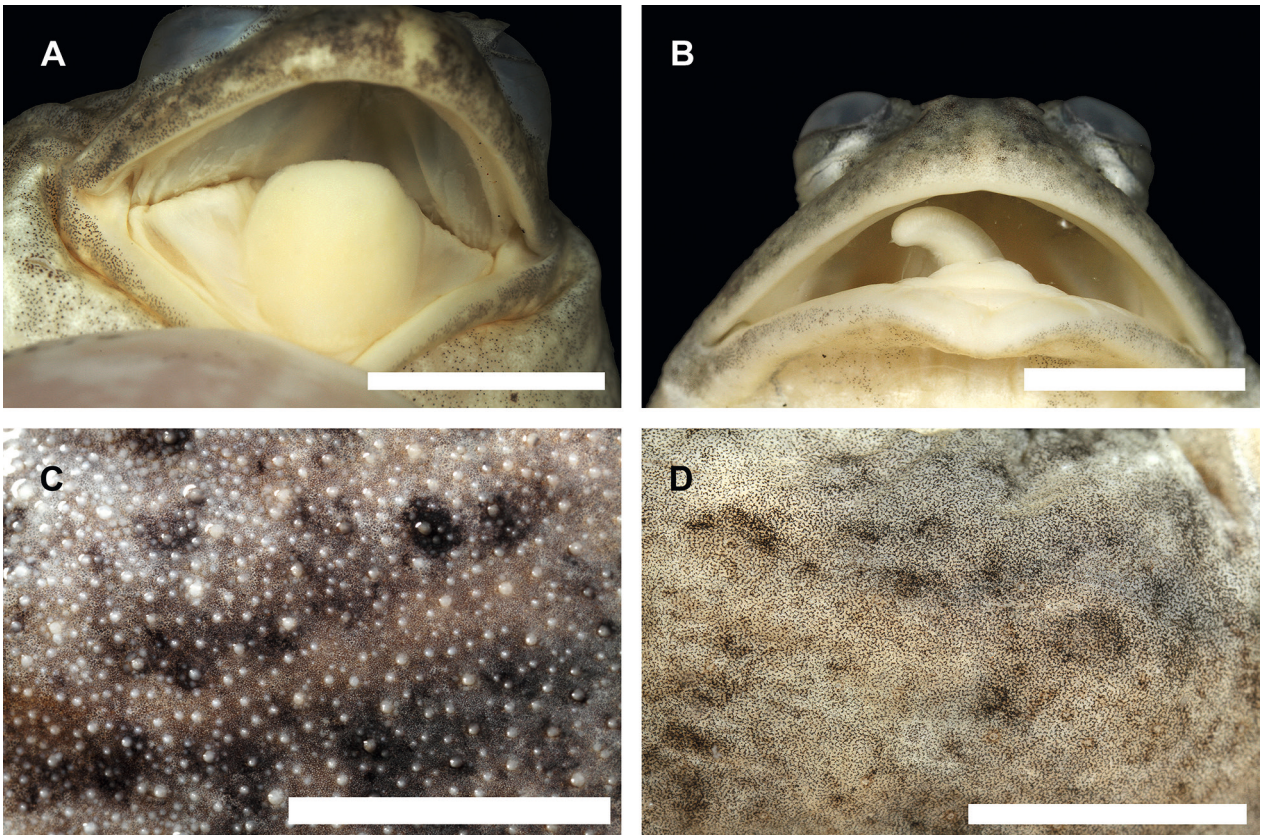

Figure 7. Morphological differences between *Phrynoglossus* and *Occidozyga*. Tongue morphology in (A) *P. myanhessei* **n. sp.**, SMF 103353; (B) *O. lima*, GK-7076; dorsal skin texture in (C) *P. myanhessei* **n. sp.**, SMF 103353; (D) *O. lima*, GK-7076. Photos by G.K.

Phrynoglossus myanhessei Köhler, Vargas, Than & Thammachoti sp. nov.

http://zoobank.org/4D51EF6E-E61D-4A91-90EC-56B160400126

Figs. 8–10

Holotype. SMF 103841, an adult male collected at East Yangon University (16.77737, 96.24065; 17 m a.s.l.), Thanlyin, Yangon, Myanmar, collected 6 July 2017 by Gunther Köhler and Ni Lar Than. Field tag number GK-6728.

Paratypes. SMF 103840, same collecting data as holotype; SMF 103797–99, same locality data and collectors as holotype but collected 15 June 2018. All paratypes are adult males except for SMF 103798 which is an adult female.

Diagnosis. A species of the genus *Phrynoglossus* as defined above that differs from all mainland Southeast Asian congeners by having (1) a large body size (males 22.0–27.4 mm; females 28.4–31.3 mm); (2) relatively small tympanum (ratio TYD/SVL 0.48–0.92); and (3) call duration of male advertisement call 85–114 ms. *Phrynoglossus myanhessei* differs from its congeners in Indochina (i.e., *P. martensii* and *P. magnapustulosus*) in the male advertisement call, most obvious in call duration (85–114 ms in *P. myanhessei*, 32–52 ms in *P. martensii*, 243–437 ms in *P. magnapustulosus*) and dominant frequency (2454–2885 Hz in *P. myanhessei*, 3338–3962 Hz in *P. martensii*, 3187–3790 Hz in *P. magnapustulosus*).

Comparisons. *Phrynoglossus myanhessei* differs from its congeners as follows (condition for *P. myanhessei* in parentheses). *Phrynoglossus floresianus*, *P. semipalmatus*, and *P. tompotika* all have distinctly enlarged flattened toe and finger disks (tips of toes rounded, only slightly expanded into discs, not distinctly flattened); *P. floresianus* and *P. laevis* are much larger frogs, 35–37 mm in males, 40–51 mm in adult females in *P. floresianus*, 26–42 mm in males, 35–62 mm in females in *P. laevis* (males 22.0–27.4 mm; females 28.4–31.3 mm); furthermore, the eyes are directed dorsolaterally in *P. laevis* and *P. celebensis* (laterally); *P. baluensis* usually has a large inverted U-shaped ridge on the dorsum (no such ridge present) and reduced toe webbing with at least two phalanges free of webbing along fourth toe (feet almost fully webbed, less than one phalange free of webbing along fourth toe); *P. sumatranus* has a dark brown band on each side of the cloaca (such band absent), diamond shaped pupil (ovoid), and its eyes are oriented dorsolaterally (laterally); *P. diminutivus* has reduced toe webbing with two phalanges free of webbing along fourth toe (feet almost fully webbed, less than one phalange free of webbing along fourth toe); *P. martensii* and *P. magnapustulosus* are smaller frogs, 20–23 mm in males, 26–30 mm in adult females in *P. martensii*, 17–20 mm in males, 17–24 mm in females in *P. magnapustulosus* (males 22.0–27.4 mm; females 28.4–31.3 mm); furthermore, *P. magnapustulosus* has a relatively larger tympanum, ratio tympanum/SVL 0.055–0.099, mean 0.082 (0.029–0.066, mean 0.050). Also, *P. martensii* has a relatively broader head, ratio HW/SVL 0.336–0.401, mean 0.357 in males, 0.338–0.388, mean 0.351 in females (0.286–0.369, mean 0.338 in males, 0.283–0.354, mean 0.312 in females).

Description of the holotype (Figs. 8 and 9). Adult male, as indicated by dark colored throat region and presence of vocal slits; SVL 23.56 mm; habitus robust; head broad, about as wide as long, ratio HL/HW 1.06; 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 0.12; IOD (2.12) greater than width of upper eyelid (1.94); tympanum concealed, slightly depressed relative to skin of temporal region, tympanic rim weakly elevated relative to tympanum; ratio TYD/EYD 0.42; vomerine teeth absent; tongue fleshy, rounded, without notch; tips of all four fingers rounded, not expanded into discs; relative finger lengths III>I>IV>II; no webbing; distinct subarticular tubercles, palmar tubercle distinct, bifid; thenar tubercle large, 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 0.8–0.8 II 0.8–0.8 III 0.8–0.9 IV 0.9–0.8 V; a well-developed flap of skin on postaxial side of Toe V from level of outer metatarsal tubercle to distal subarticular tubercle; strong fold on distal one-half of tarsus; large, flap-like inner metatarsal tubercle; outer metatarsal tubercle not differentiated, but rather two tiny tubercles present in that area; skin on top of head and on dorsum and flank smooth; skin on throat and venter shagreen; skin on upper surface of forelimbs and thigh with low, rounded tubercles, that of dorsal surface of shank with scattered keratinized pointed tubercles; indistinct, glandular supratympanic fold 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 23.56; HL 8.91; HW 8.44; SL 3.60; EYD 2.81; IOD 2.12; TYD 1.17; TED 0.67; SHL 12.51; THL 12.52; HNL 6.04; FL 12.22; NED 2.37; IND 1.92.

Coloration in life was recorded as follows (Fig. 10): Dorsal and lateral ground color of head and body Drab-Gray (256) with indistinct Raw Umber (280) blotches and mottling; dorsal surfaces of forelimbs True Cinnamon (260) with Raw Umber (280) speckles; dorsal surfaces of hind limbs Drab-Gray (256) with Raw Umber (280) transverse broken bars; a Drab-Gray (256) oblique bar from anterior corner of eye to snout; throat region heavily suffused with Vandyke Brown (282); venter Pale Neutral Gray (296) with Smoky White (261) stipples; ventral surfaces of forelimbs Medium Fawn Color (257); ventral surfaces of hind limbs Cream White (52); iris Olive Brown (278) with a suffusion of Orange-Rufous (56) above and whitish below.

Coloration after about three years preservation in 70% ethanol was recorded as follows: Dorsal and lateral ground color of head and body Glaucous (272) with indistinct Sepia (279) blotches and mottling; dorsal surfaces of hind limbs Glaucous (272) with Sepia (279) transverse

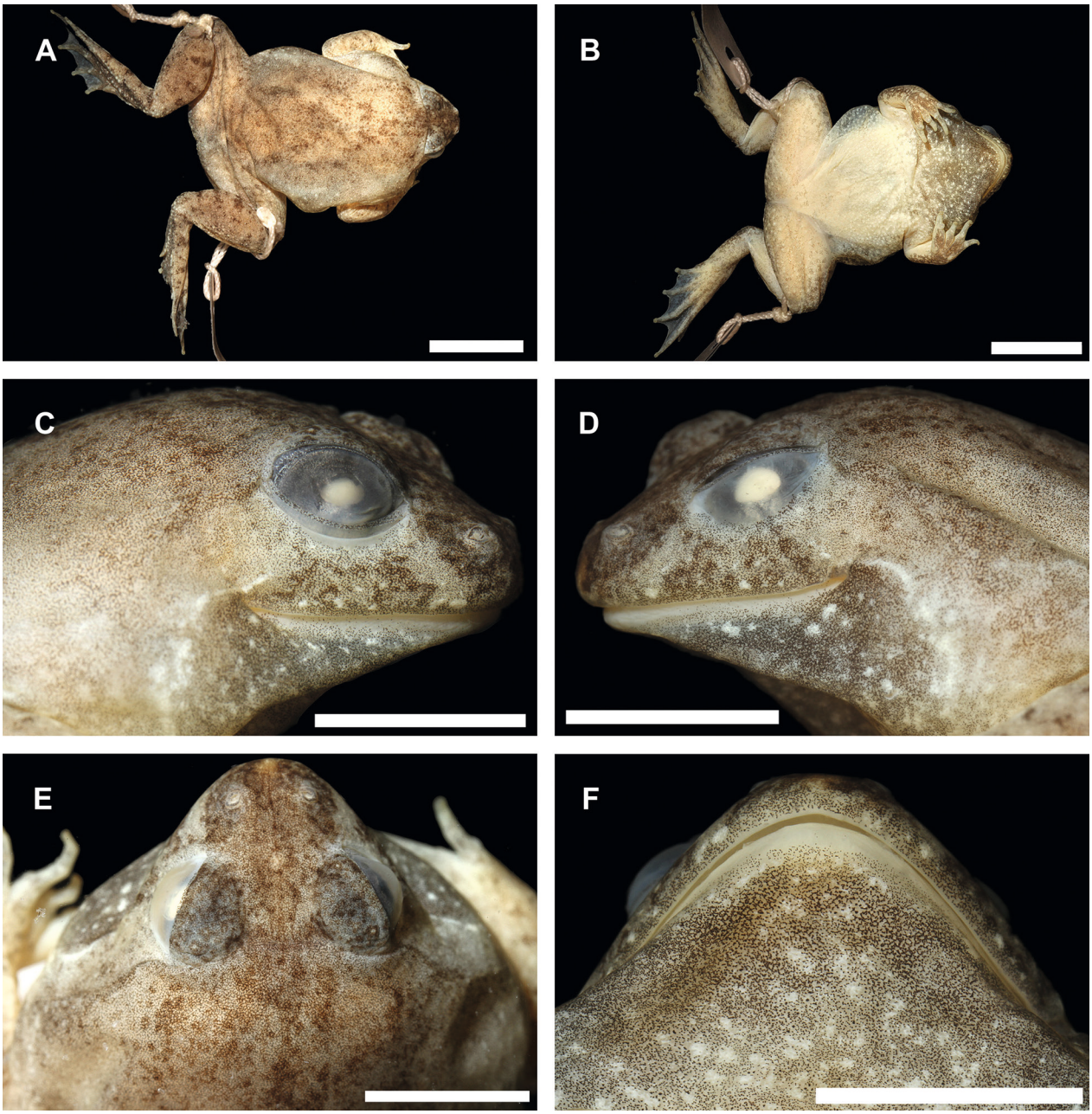

Figure 8. Holotype of *Phrynoglossus myanhessei* **n. sp.** (SMF 103841). Scale bars equal 10.0 mm (A, B) and 5 mm (C–F). Photos by G.K.

broken bars; a Drab-Gray (256) oblique bar from anterior corner of eye to snout; supraocular region Dusky Brown (285); throat region Glaucous (289) with Pale Buff (1) stipples; venter Pale Buff (1) with Smoky White (261) stipples; ventral surfaces of forelimbs Glaucous (289); ventral surfaces of hind limbs Light Buff (2).

Variation. The paratypes agree well with the holotype in general appearance; morphometrics and coloration (see Table 3).

Etymology. "Myan" is Myanmar's abbreviated name and was chosen because this species is endemic to Myanmar as far as we know. "hessei" was chosen in recognition of the long-term support and funding of Senckenberg by the German State of Hesse. In combination, the species name *myanhessei* reflects the long-term productive collaboration of researchers from Hesse and Myanmar in the field of herpetology.

Natural history notes. At the type locality, the specimens were collected at night in a patch of muddy grass area, partly open, partly covered by bushes and low trees. The frogs were sitting at the edge of small shallow temporary water bodies.

Geographic Distribution and Conservation. As currently known, *Phrynoglossus myanhessei* is restricted to central and lower Myanmar (Fig. 11). This species was abundant wherever we found it. Thus, we classify it as Least Concern according to the IUCN categories (IUCN, 2012).

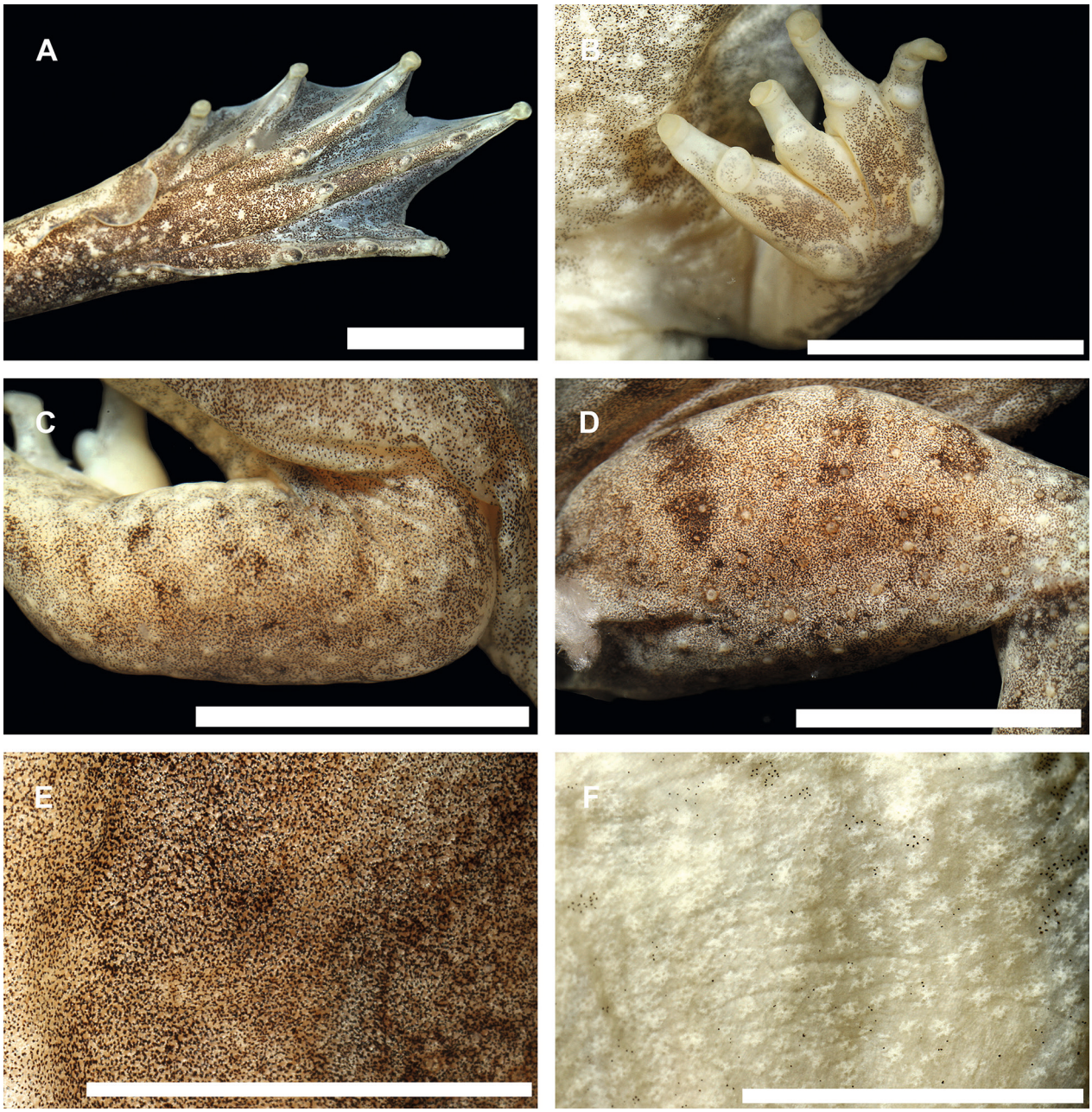

Figure 9. Holotype of *Phrynoglossus myanhessei* **sp. n.** (SMF 103841). Scale bars equal 5.0 mm. Photos by G.K.

Genomic characterization. Whole genome sequencing and assembly: Illumina sequencing yielded 616,151,068 short-reads with a data amount of 92.4 Gb. K-mer analysis estimated the genome size to 2.6 Gb and heterozygosity 0.9%. The mitochondrial genome was assembled into one circular sequence with a length of 18,348 bp (accession number MW405414). All expected 13 protein coding genes, 2 rRNAs, 23 tRNAs, one D-loop region and the origin of replication could be annotated on the mitochondrial genome sequence. A pairwise alignment to the complete mitochondrial genome of *Phrynoglossus martensii* (GU177877) shows 77.1% pairwise identity. The final nuclear genome assembly contains 1,446,664 contigs with a total length of 1,829,122,027, an N50 of 1,468 and a GC of 41.07%. The backmapping rate is as high as 98.9% and after filtering, the genome sequence coverage of the holotype specimen is uniformly distributed at 25×. The BUSCO search resulted in 20.8% present BUSCOs (C:8.5%[S:8.4%,D:0.1%],F:12.3%,M:79.2%,n:5310) and no contamination could be identified by interpreting the blobplot. Raw reads and the draft-genome assemblies can be found within the BioProject PRJNA687006. For further details see Appendix 3.

Discussion

Our study provides support for the general assumption that most geographically wide-spread species contain cryptic diversity with one or more unrecognized species "hiding" behind another taxon name due to its similarity in external morphology (Hasan et al. 2012; Köhler et al.

Figure 10. *Phrynoglossus myanhessei* **sp. n.** in life. (A) Male holotype, SMF 103841; (B) male paratype, SMF 103840; (C) male paratype, SMF 103799; (D) female paratype, SMF 103798. Photos by G.K.

2019). Typically, these anuran species differ from one another in bioacoustics, i.e., in the male advertisement call. Often these species are poorly differentiated in external morphology which was the reason for lumping them before in the first place. The reason for the poor degree of morphological differentiation is possibly the lack of ecological differentiation of the members of such a cluster of frogs. In the case of the *Phrynoglossus* studied here, it is most likely that they went through the process of speciation in allopatry as indicated by their present distribution pattern. A single prezygotal isolating mechanism is enough to finalize the speciation process no matter how similar the diverging populations are in other characters such as external morphology or ecological traits, e.g., habitat preference, diet, or activity patterns (Wilson, 2001). In anurans the male advertisement call serves as a very effective isolating mechanism to avoid hybridization among similar species under natural conditions (Köhler et al. 2017). Thus, the discovery that there are three distinct male advertisement calls among the species referred to as *P. martensii* in mainland Southeast Asia is strong evidence for the presence of three distinct species that are not compatible reproductively. In this sense it could be expected that the variation in mtDNA data would be congruent with the results of the bioacoustical analyses. The mtDNA data found a fourth clade of frogs (our Clade 2 with specimens from northern Thailand, southern China, Vietnam, Laos, and Cambodia) that differed from the other three clades by a mean genetic distance of 16S of 3.5–5.1% and therefore qualifies as candidate species (sensu Fouquet et al. 2007). However, since we did not find additional support for the recognition of this clade as a distinct evolutionary species, we tentatively assigned it to the species it is genetically most closely related too (i.e., *P. martensii*). Additional research including nuclear genetic data is needed to evaluate these northeastern populations.

In previous publications, the geographic distribution of *Phrynoglossus magnapustulosus* was given as "Nakhon Phanon, Ubon, Loei, and Chiang Mai provinces, Thailand" (e.g., Taylor 1962; Frost 2020) or "northern and northeastern Thailand" (Khonsue and Thirakhupt 2001). According to our molecular genetic data and also based upon our field observations in Chiang Mai, this statement is erroneous and probably based on misleading characters of external morphology that were considered to be diagnostic for this species such as the presence of "pearly tubercles" on the dorsum (Taylor 1962). In our series of topotypic specimens of *P. magnapustulosus* we found that dorsal skin texture varies considerably. Some specimens virtually have only a few low tubercles on the dorsum whereas other have distinct ones, pearl-tipped or not (see Fig. 12). A similar degree of variation in dorsal skin texture was observed in individuals of *P. martensii* (Fig. 13). Thus, we dismiss this character as useful for distinguishing among species of *Phrynoglossus*. As far as known, *P. magnapustulosus* is restricted to the Khorat Plateau. Other herpetofaunal species endemic to the Khorat Plateau include *Enhydris subtaeniata* and *Malayemys khoratensis* (Murphy and Voris 2014; Ihlow et al. 2016).

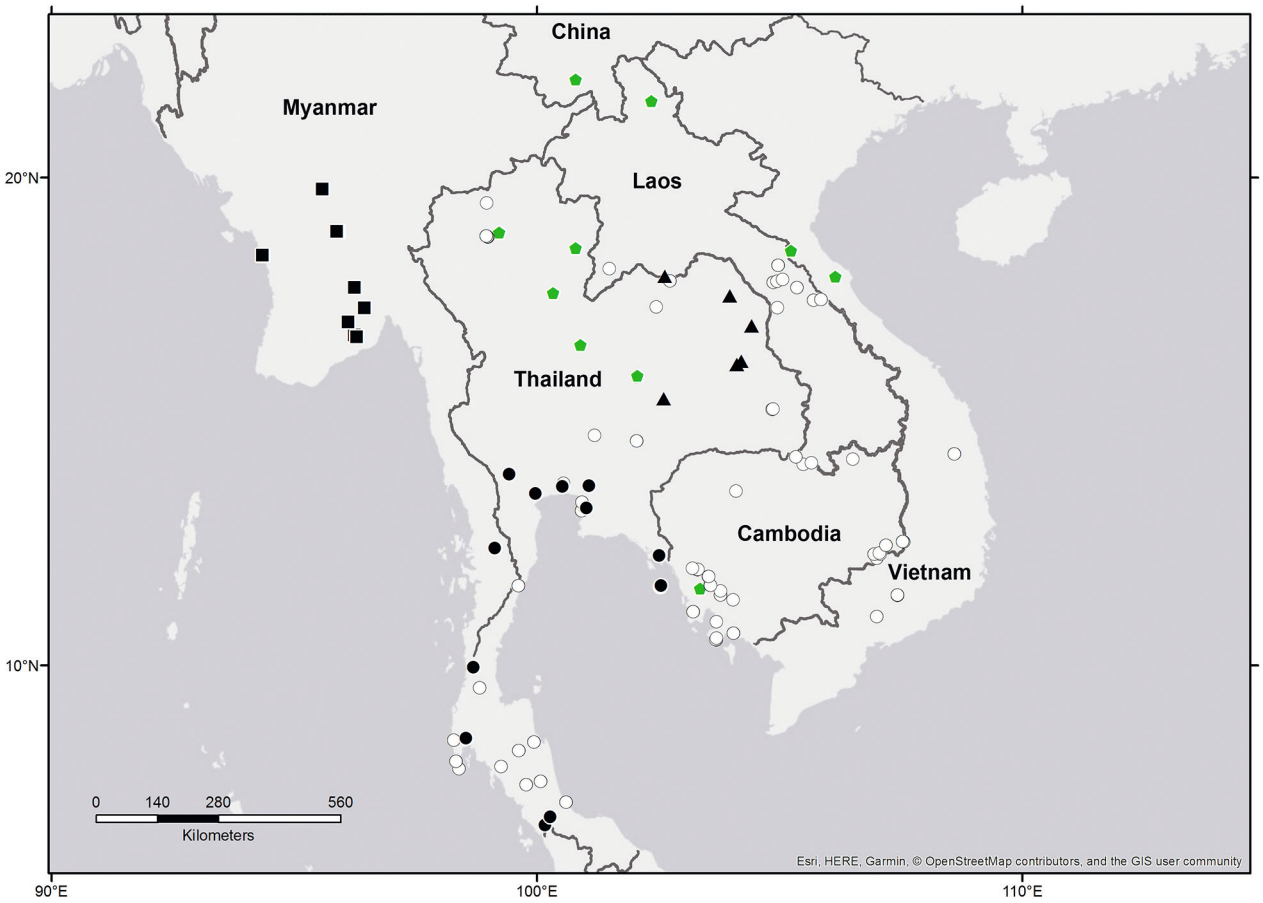

Figure 11. Map indicating collecting localities of the *Phrynoglossus* species occurring in Indochina. Each symbol can represent one or more adjacent localities. Black squares: *P. myanhessei* **n. sp.**; black circles: *P. martensii*; green pentagons: *P.* cf. *martensii*; black triangles: *P. magnapustulosus*; white circles: additional specimens of *Phrynoglossus* from the FMNH, not examined by authors.

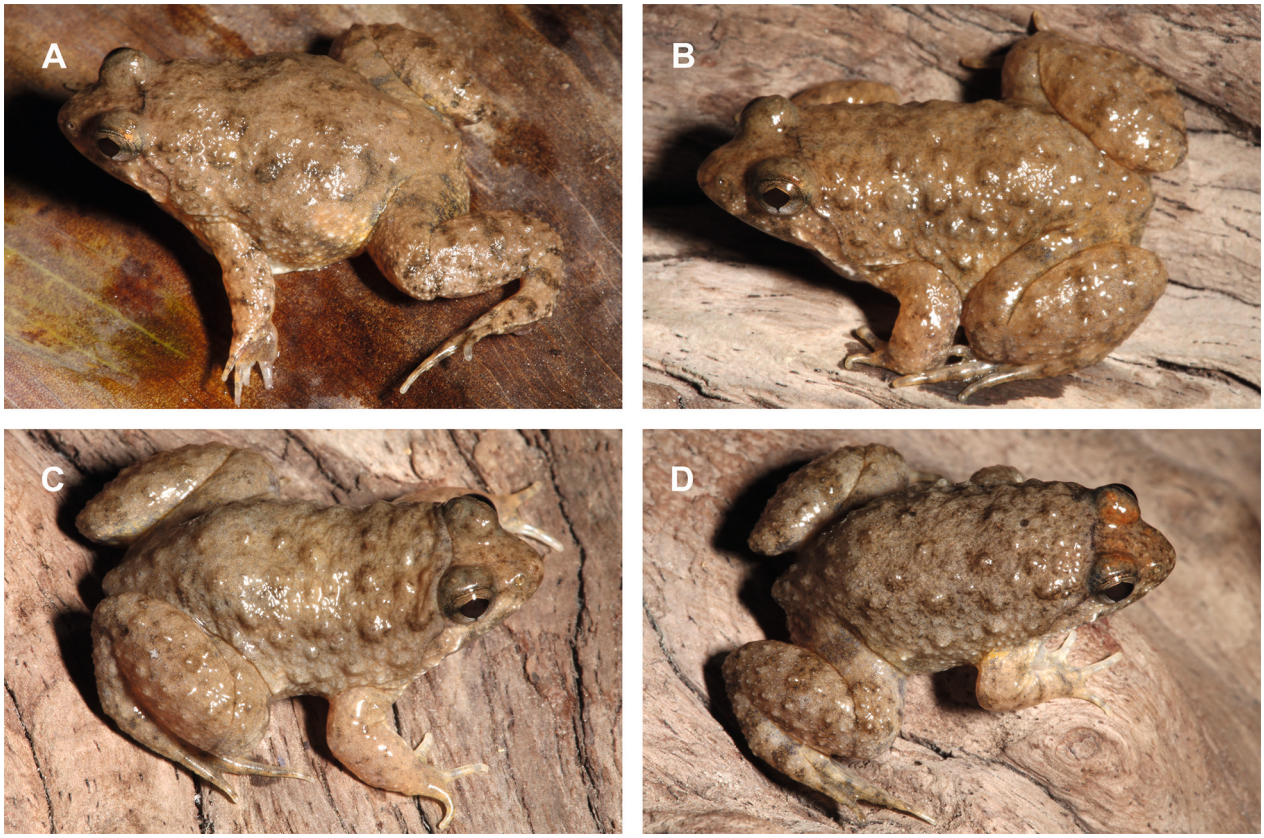

Figure 12. *Phrynoglossus magnapustulosus* in life. (A) GK-7855; (B) GK-7853; (C) GK-7877; (D) GK-7880. Photos by G.K.

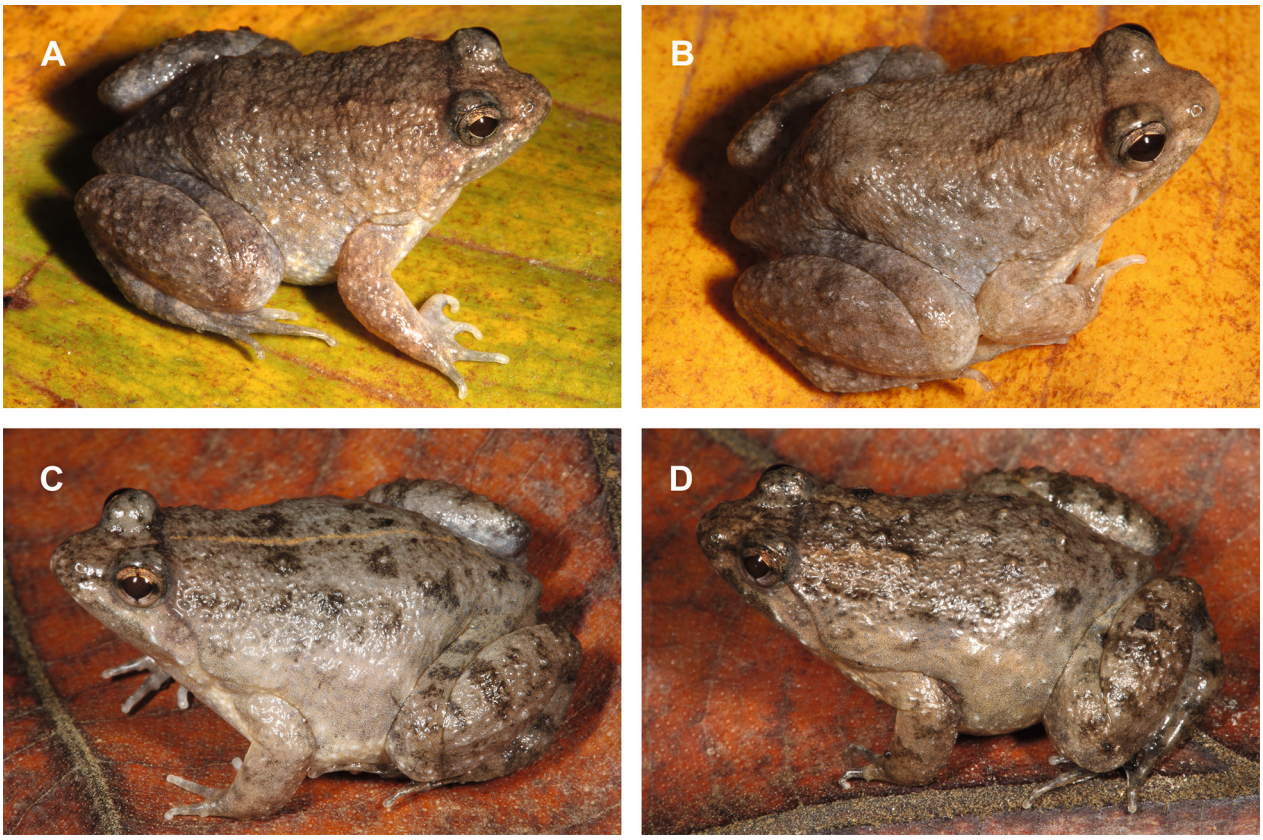

Figure 13. *Phrynoglossus martensii* in life. (A) GK-7350; (B) GK-7349; (C) GK-7235; (D) GK-7237. Photos by G.K.

The species of *Phrynoglossus* (or *Occidozyga*) that are supposedly endemic to the islands of the Sunda Archipelago and the Philippines were beyond the scope of this study. Future studies need to address the phylogenetic relationships and verify their generic assignment as proposed here. The integrative taxonomic approach including also bioacoustic and genetic data along with a thorough analysis of the geographic variation of external morphology will be useful in order to clarify the taxonomic status of these populations.

The evidence for recognizing *Phrynoglossus* and *Occidozyga* as distinct evolutionary units at genus level is supported by the monophyly of the two taxa based on the analyses of mtDNA in this study. Furthermore, there are several diagnostic morphological characters that define each genus, some of which may turn out to be apomorphic for one or the other genus. The conspicuous skin morphology of *Occidozyga* as well as its unique tongue shape are such candidates. Regarding the documented mode of amplexus, finding two different modes in these supposedly closely related genera is interesting. The inguinal amplexus mode that we documented in *P. magnapustulosus* and *P. myanhessei* is supported as an autapomorphy of the genus *Phrynoglossus* by the observation of an inguinal amplexus mode also in *P. sumatranus* by Eto and Matsui (2012). According to a large-scale analysis of the amplexus mode across all groups of anuran amphibians, reveal the inguinal amplexus as the basal state to all Anura (Carvajal-Castro et al. 2020). However, the latter authors gave the axillary amplexus as the only mode found within the family Dicroglossidae obviously not being aware of the inguinal amplexus mode in *Phrynoglossus*. Given the phylogenetic position of *Phrynoglossus* in the amphibian tree of life (Pyron and Wiens 2011), we interpret the amplexus mode in this genus as a reversal from the axillary mode, and thus as an autapomorphy of this genus.

With this description of a new species we provide the complete, annotated mitochondrial genome, a draft genome and 25× coverage of short-read genome resources of the holotype. This fundamental genomic characterization of the species based on the name-bearing specimen will be an invaluable genomic resource for future taxonomic and evolutionary studies. The quality of the nuclear genome assembly provided for the holotype of *Phrynoglossus myanhessei* is sufficient to genetically characterize the new species on the basis of the name-bearing specimen. The genome data describe the entire genetic variation, including heterozygosity of the holotype individual and make it possible to extract any genetic locus for future taxonomic or phylogenetic studies specifically of occidozygine frogs and frogs in general.

Following the first example from Köhler et al. (2021) we reiterate our proposition that wherever feasible, descriptions of new species should be accompanied by genomic data and a draft genome assembly from the respective holotype as an important resource to future biodiversity research. The investment in a draft genome of newly described holotypes ensures that the genetic fingerprint of newly described species is captured and preserved long-term based on the name-bearing specimen. Furthermore, Köhler et al. (2021) suggest to balance between quality and investment regarding draft genome quality.

Therefore the quality of the nuclear genome assembly from 25× coverage is not very high by purpose and still provides a basis for further studies. In addition the complete mitochondrial genome is a valuable resource e.g., for further phylogenetic studies. In the near future, adding genomic data to traditional phenotypic studies will likely become a standard, as costs and efforts to produce them drop dramatically. And the time to start is now.

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Appendix 1

Comparative Specimens Examined

Occidozyga lima—**China:** Guangdong: no further data: SMF 6571–72; Lo-fou-shan Mountains: SMF 6573; Hainan: SMF 6570. **Indonesien:** Java: no further data: SMF 6574–75; Weltevreden: SMF 23436. **Myanmar:** Bago: Bago Yoma, 125 m: GK-7075–77; Ko Pya Gyi, 55 m: GK-7072; Naypyidaw: near Yamethin, 206 m: SMF 103817–19; Yangon: East Yangon University, 65 m: GK-7057, 7733; near Taw Hlan village: 1: SMF 103815–16. Thailand: Nakhon Phanom: Ban Kan Luang, 173 m: GK-7881–82; Nakhon Ratchasima: near Lake Resort Khorat, 142 m: GK-7917, 7920; Rayong: near Rayong, 110 m: GK-7721; Roi Et: near Ban Sa At Na Di, 160 m: GK-7409–10, 7415–17, 7451–52, 7477–79, 7538–40; near Selaphum, 130 m: GK-7794; Sakon Nakhon: Ban Phaeng Yai: 155: GK-7526. **Thailand:** Yasothon: 13 km NE Ban Sa At Na Di: 202: GK-7469.

Phrynoglossus celebensis—**Indonesia:** Sulawesi Selatan: Djikoro, Mt. Bouthain: SMF 16332.

Phrynoglossus floresianus—**Indonesia:** Flores: Nusa Tenggara Barat, Rana Mese: SMF 23438–45.

Phrynoglossus laevis—**Philippines:** Laguna: Mt. Makiling, Los Banos: SMF 74674; Leyte: no further data: SMF 6591; Mt. Balocaue, Baybay: SMF 74614; Luzon: Manila: SMF 6578–80, 6587; Central Luzon: SMF 6581–86; Masbate: Panal: SMF 74317–30; Negros Oriental: Lake Balinsassyao: SMF 75006; Palawan: Calauit Island, northwestern peak of the Busunga: SMF 74134, 74437; Tarusan: SMF 74421–33; Quirino: Sierra Madre: SMF 74637–39; Samar: Hinabangen: SMF 75202–04.

Phrynoglossus myanhessei—**Myanmar:** Bago: Bago Yoma, 425 m: GK-7104–05, 7139; Bago, 50 m: SMF 103351–53; Magwe: near Taungdwingyi, 170 m: SMF 103800–03; Rakhaing: Ngapali Mountains, 50 m: SMF 103843; Ngapali, Dam Lake, 10 m: SMF 103842; Yangon: East Yangon University, 17 m: SMF 103840–41, 103797–99.

Phrynoglossus magnapustulosus—**Thailand:** Nakhon Phanom: Ban Kan Luang, 173 m: GK-7853, 7855, 7876–80, 7887; Nakhon Ratchasima: near Lake Resort Khorat, 142 m: GK-7915–16; Roi Et: near Ban Sa At Na Di, 145 m: GK-7361, 7465, 7496–503, 7805–06, 7395–96, 7412–14, 7475–76, 7509, 7513–16, 7537; Sakon Nakhon: Ban Phaeng Yai, 155 m: GK-7532–34; Yasothon: 13 km NE Ban Sa At Na Di, 202 m: GK-747071.

Phrynoglossus martensii—**Thailand:** Bangkok: Bangkok, 3 m: PT-2634; Chiang Mai: Huai Hong Khrai Royal Development Study Centre, 430 m: GK-7234–35, 7237–38, 7249, PT-1543–441771, 2076; Chaiyaphume: Ban Na Si Nuan, 205: PT-0167–68; Chonburi: Ya Teng Homestay, 30 m: GK-7349–50; Kanchanaburi: Klon Do, Dan Makham Tia District, 33 m: PT-2644–45; Nakornratchasima: Wang Nham Khiau, 515 m: PT-0033–36, 0038–39; Nan: near Klang Wiang, 257 m: PT-2669–70, 2672–73; Prachuap Khiri Khan: Ko Thalu, 8 m: PT-0535; Ratchaburi: Damnoen Saduak, 5 m: GK-7365; Trat: Ko Kut Resort, 35 m: GK-7713–14, PT-1025; Trat, 5 m: GK-7695–701; Satun: near Masayit Gi Ma Min Din, 102 m: PT-2852–53; Songkhla: Wang Pha, 98 m: PT-2754–59.

Phrynoglossus sumatranus—**Indonesia:** Bali: Batoeriti: SMF 23454–55; Gitgit: SMF 23447–53; Java: Jawa Barat: Bogor: SMF 6576–77, 23437, 31165–68; Jawa Tengah: Wonosobo: SMF 31229; Jawa Timur: Punten: SMF 31230–34; Bujutan at Ardjasa, northwestern coast of P. Kangean, Kangean Islands: SMF 55307–08; Sulawesi: no further data: SMF 6600; Sulawesi Utara: Minahasa: SMF 6598–99; Sumatra: Stabat, Deli: SMF 6588–90.

Appendix 2

Genbank accession numbers for the 16S sequences used in this study

Species	*Specimen number*	**GenBank number**
Ingerana tenasserimensis	USNM 586923	MG935841
Ingerana tenasserimensis	USNM 587300	MG935839
Ingerana tenasserimensis	USNM 587302	MG935840
Ingerana tenasserimensis	USNMFS35684	MG935838
Limnonectes limborgi	GK_7110	MW217495
Occidozyga lima		AB530619
Occidozyga lima	GK_7409	MW217509
Occidozyga lima	GK_7451	MW217508
Occidozyga lima	GK_7452	MW217507
Occidozyga lima	GK_7721	MW217498
Occidozyga lima	GK_7733	MW217497
Occidozyga lima	GK_7794	MW217496
Occidozyga lima	GK_7924	MW217506
Occidozyga lima	ROM 25003	AF206497
Occidozyga lima	SMF 103815	MW217492
Occidozyga lima	SMF 103817	MW217494
Occidozyga lima	SMF 103818	MW217493
Occidozyga lima		KR827958
Occidozyga lima		KR827959
Occidozyga lima		KR827960
Phrynoglossus myanhessei	SMF 103797	MW217501
Phrynoglossus myanhessei	SMF 103798	MW217502
Phrynoglossus myanhessei	SMF 103800	MW217503
Phrynoglossus myanhessei	SMF 103840	MW217499
Phrynoglossus myanhessei	SMF 103841	MW217500
Phrynoglossus myanhessei	USNM 587105	MG935916
Phrynoglossus myanhessei	USNM 587107	MG935920
Phrynoglossus myanhessei	USNM 587395	MG935918
Phrynoglossus myanhessei	USNM 587402	MG935917
Phrynoglossus magnapustulosus		KR827981
Phrynoglossus magnapustulosus	GK_7395	MW217488
Phrynoglossus magnapustulosus	GK_7396	MW217487
Phrynoglossus magnapustulosus	GK_7532	MW217486
Phrynoglossus magnapustulosus	GK_7533	MW217485
Phrynoglossus magnapustulosus	GK_7855	MW217490
Phrynoglossus magnapustulosus	GK_7916	MW217489

Appendix 2 continued.

Species	*Specimen number*	GenBank number
Phrynoglossus martensii		AF285214
Phrynoglossus martensii		GU177877
Phrynoglossus martensii		KP318725
Phrynoglossus martensii		KR827982
Phrynoglossus martensii		KR827983
Phrynoglossus martensii		KR827984
Phrynoglossus martensii		KR827985
Phrynoglossus martensii	AM07357	NC_014685
Phrynoglossus martensii	AMNH A161171	DQ283357
Phrynoglossus martensii	GK_7349	MW217491
Phrynoglossus martensii	GK_7695	MW217504
Phrynoglossus martensii	GK_7713	MW217505
Phrynoglossus martensii	PT_0167	MW217484
Phrynoglossus martensii	PT_0600	MW217481
Phrynoglossus martensii	PT_0942	MW217480
Phrynoglossus martensii	PT_0943	MW217479
Phrynoglossus martensii	PT_1172	MW217478
Phrynoglossus martensii	PT_1543	MW217477
Phrynoglossus martensii	PT_1544	MW217476
Phrynoglossus martensii	PT_2634	MW217475
Phrynoglossus martensii	PT_2754	MW217483
Phrynoglossus martensii	PT_2755	MW217482
Phrynoglossus martensii	ROM 22222	AF206467
Phrynoglossus martensii	SCUM0437980	DQ458254
Phrynoglossus martensii	SCUM0437983	DQ458255
Phrynoglossus martensii	SCUMH020	DQ458256
Phrynoglossus martensii	TAD P324	KR827986
Phrynoglossus martensii	USNM 586940	MG935942
Phrynoglossus martensii	USNM 586941	MG935929
Phrynoglossus martensii	USNM 586942	MG935941
Phrynoglossus martensii	USNM 586943	MG935940

Appendix 3

Genomics

Material and Methods. Default parameters are applied, if not stated otherwise.

Raw data and preprocessing. A *k*-mer profile was created from the raw data using Jellyfish 2.3.0 (Marçais & Kingsford, 2011) including the parameters "***-F 2 -C -m 21 -s 1000000000 -t 96***" for ***count*** and "***-t 96***" for ***histo***. The resulting histogram was uploaded to the GenomeScope webserver (Vurture et al., 2017) to retrieve certain statistics of the genome.

Low quality bases and adapter sequences were trimmed from the raw reads using Trimmomatic's 0.39 (Bolger et al., 2014) paired end mode along with the options to create a summary and "***-threads 96***". For adapter trimming all adapter sequences provided within Trimmomatic were used. The following trimmers were set: "***ILLUMINACLIP:<adapter_all.fa>:2:30:10:8:true SLIDINGWINDOW:4:20 MINLEN:50 TOPHRED33***".

To filter out reads originating from possible contamination, Kraken 2.0.9 (Wood et al.,2019) was ran with a standard database built on March 18th, 2020 (including "complete genomes in RefSeq for the bacterial, archaeal, and viral domains, along with the human genome and a collection of known vectors (UniVec_Core)") and the paired and unpaired trimmed reads as input. For both runs the options "***--threads 96 --unclassified-out <out-file(s)>***" were set and for the paired run the option "***--paired***".

Nuclear genome assembly and quality control. To estimate the best length of *k* for genome assembly, KmerGenie 1.7051 (Chikhi & Medvedev 2014) was applied, using the raw data as input and the options "***--diploid -s 11 -k 141 -t 60***".

The unclassified paired and unpaired reads were assembled using Velvet 1.2.10 (Zerbino & Birney 2008) by

first running ***velveth*** for a *k*-mer length of 61 and second running velvetg with the options "***-cov_cutoff auto -ins_length 350 -min_contig_lgth 500***".

The quality of the resulting scaffolds was tested by a) mapping the reads used for assembly back to the assembly, b) checking for possible contamination and c) searching for expected orthologous genes. Backmapping was performed with backmap.pl 0.3 (https://github.com/schellt/backmap), which utilized bwa mem 0.7.17-r1188 (Li, 2013), samtools 1.10 (Li et al., 2009), Qualimap 2.2.1 (Okonechnikov et al., 2016), bedtools 2.28.0 (Quinlan & Hall, 2010) and R 4.0.2 (R Core Team, 2020). Contamination screening on the assembly level was performed with blobtools 1.1.1 (Laetsch & Blaxter, 2017). The bam file resulting from the above mentioned backmapping was coverted to a blobtools readable cov file by first indexing the bam file with samtools index and second converting with blobtools map2cov. To assign Taxonomy IDs blastn 2.10.0+ (Camacho et al., 2009) was used to align the scaffolds against the complete nt database (***-task megablast -outfmt '6 qseqid staxids bitscore' -evalue 1e-25 -num_threads 96***). From the cov and hits file a blobDB was created and plotted. Completeness in terms of core orthologs was screened with BUSCO 4.1.4 (Simão et al., 2015) along with the tetrapoda_odb10 set and the options "***-c 8 -o GK_6728_velvet -m geno --long --offline***"

Mitochondrial genome assembly and annotation. NOVOplasty 4.2 (Dierckxsens et al., 2017) was used along with the longest annotated gene (ND5; ACZ02636.1) from *Occidozyga martensii* (GU177877.1) as seed. The config file was changed compared to the included one for the options "Max memory = 100; Read Length = 150; Insert size = 300" and the options "Reference sequence, Chloroplast sequence" were left blank. As input reads the untrimmed raw data was used as recommended. The mt genome assembly was checked for consistency by alignment against the closest available mt genome of *Occidozyga martensii* (GU177877.1). This was computed with clustalo 1.2.3 (Sievers et al., 2011) in Geneious Prime 2020.2.3 (https://www.geneious.com) using Java 11.0.6+10 (***clustalo-1.2.3-Ubuntu-x86_64 -i input.fasta -o clustal.aln -v --outfmt=clustal --output-order=tree-order --iter=0 --cluster-size=100 -t DNA***). The assembled mt genome sequence was manually cut based on the alignment to fit to the cut site of the reference. The cut mt genome sequence was submitted to two mt genome annotation webservers: GeSeq (Tillich et al., 2017) and MITOS2 (Donath et al., 2019). For GeSeq the options circular and mitochondrial were chosen as well as tRNAscan-SE 2.0.6 (Chan & Lowe 2019; Chan et al., 2019) was enabled with sequence source as "vertebrate mitochondrial tRNAs". Furthermore the above mentioned mt reference Sequence (GU177877.1) was used as BLAT reference sequence. The MITOS2 settings can be found in Table A1. The annotations of GeSeq and MITOS2 were manually merged and curated in Geneious.

Assembly finalization. The final mt genome assembly from NOVOplasty was blasted against the assembly from Velvet to remove contigs representing the mt genome with blastn 2.10.0 and the options "***-num_threads 32 -outfmt '6 std slen'***". Blast reported multiple hits for 3 contigs. Either the single hit of one contig or the longest hit of one contig align completely to the NOVOplasty assembly. The only exception is one contig aligning with 43% of its 609bp length. Finally, all contigs producing blast hits were considered as mitochondiral origin and removed.

Table A1. Settings used for MITOS2.

Property	Value
Reference	RefSeq 63 Metazoa
Genetic Code	2
Proteins	TRUE
tRNAs	TRUE
rRNAs	TRUE
OH	TRUE
OL	TRUE
Circular	TRUE
Use Al Arab et al.	FALSE
E-value Exponent	2.0
Final Maximum Overlap	50nt
Fragment Quality Factor	100.0
Standard Code	FALSE
Cutoff	50.0%
Clipping Factor	10.0
Fragment Overlap	20.0%
Local only	TRUE
Sensitive only	FALSE
ncRNA overlap:	50 nt

Results

Raw data and preprocessing. Illumina NovaSeq 6000 sequencing yielded 616,151,068 reads with a data amount of 92.4Gb. The GenomeScope results are accessible via the permalink http://genomescope.org/analysis.php?code=uOlM17rRo9kkBpguBh0f. The genome size was estimated as 2.6Gb and heterozygosity to 0.9% (See Figure A1).

Raw read trimming was survived by 93.23% of the read pairs, 3.12% forward only, 2.17% reverse only and 1.49% reads were dropped. Of the trimmed paired reads 3.43% and 2.89% of the trimmed unpaired reads were classified via Kraken2.

Nuclear genome assembly and quality control. The Velvet assembly resulted in 1,446,672 contigs with a total length of 1,829,139,474 and an N50 of 1,468. The GC is at 41.07% and uniformly distributed. Of all assembled reads 98.9% could be mapped back to the assembly with a uniform distribution at 25× (Figure A2). No clear cluster representing contamination could be identified in the blobplot (Figure A3). Since the assembly is quite frag-

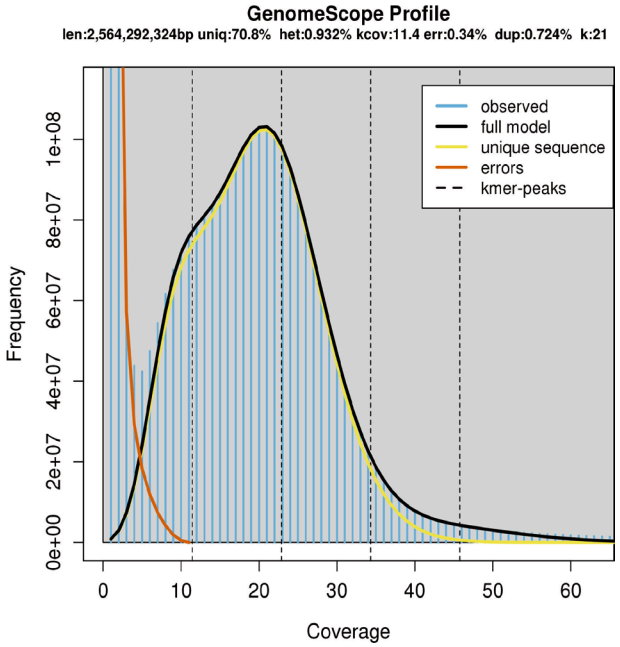

Figure A1. K-mer profile anaylsis and estimates of genome metrics of GenomeScope.

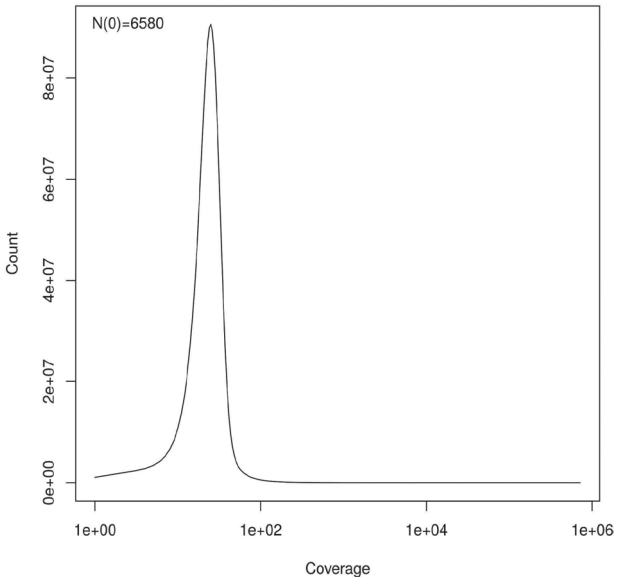

Figure A2. Coverage distribution of the assembly. A genome size estimation based on mapped nucleotides and coverage (Schell et al., 2017) with backmap.pl results in 2.83Gb, which is in line with the *k*-mer based result of 2.56Gb.

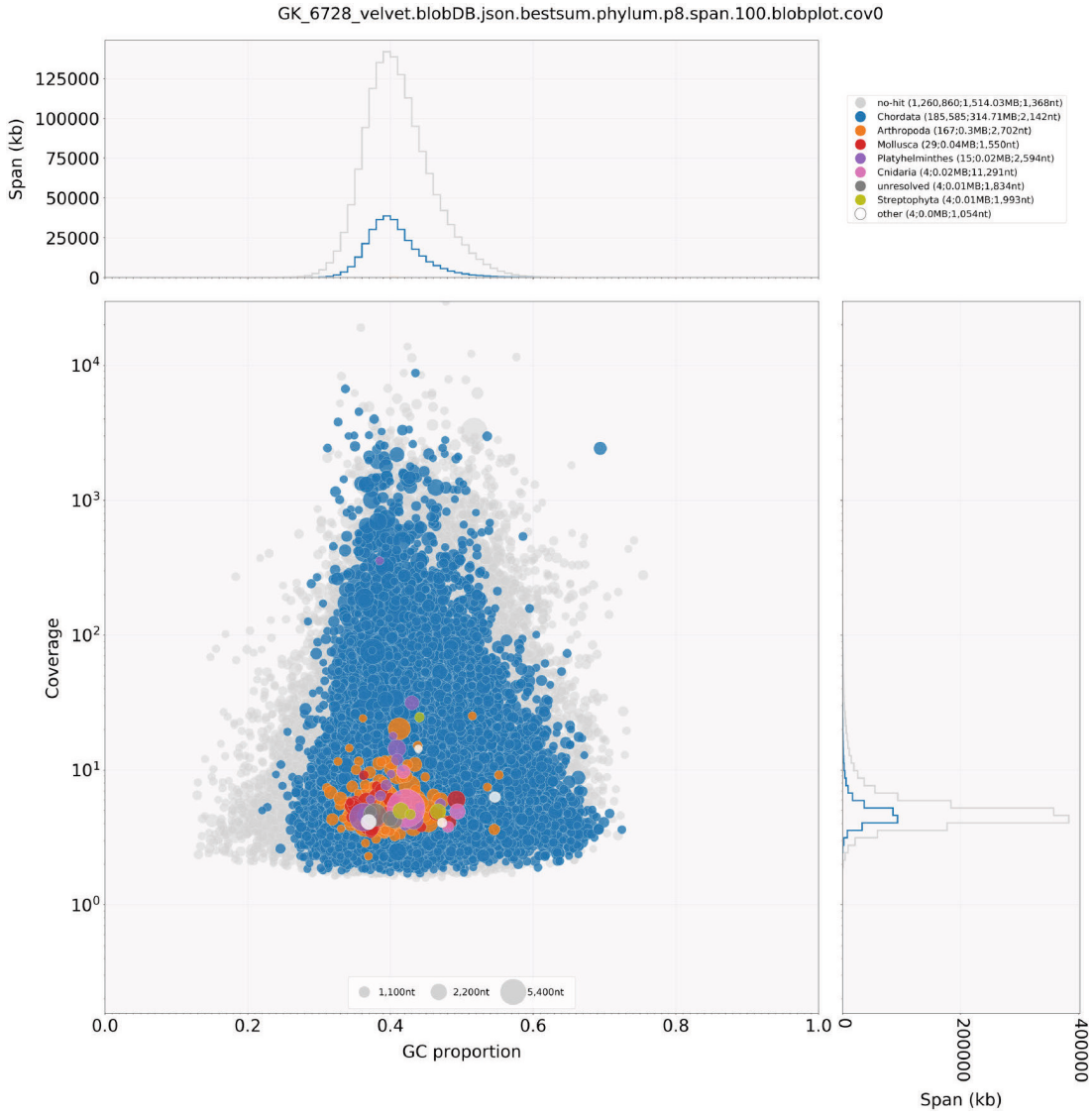

Figure A3. Blobplot of the assembly.

mented, there are no clear distinguishable clusters. Blast hits other than Chordata might arise from false positive hits and do not show enough evidence for contamination – especially because non-Chordata hits are sparse and coverage and GC of non-Chordata hits is similar to the rest. The BUSCO search resulted in C:8.5%[S:8.4%,D:0.1%],F:12.3%,M:79.2%,n:5310.

Mitochondrial genome assembly and annotation. The NOVOplasty assembly resulted in 2 contigs which could be circularized in one way. After the clustalo alignment was performed and manually adjusted by cutting the NOVOplasty assembly at the same site as the reference mt genome from *Occidozyga martensii* (GU177877.1). Since this alignment showed no huge structural differences, the assembly was used for annotation. The GeSeq annotation could not find the two rRNAs, the D-loop region and the origin of replication. The 13 protein coding genes were automatically found but with wrong reading frame and partially unlikely start and end points. In total 40 tRNAs were annotated because GeSeq does not merge the annotation from different evidences (here BLAT of reference tRNAs and tRNAscan). MITOS2 automatically annotated the two rRNAs, 13 protein coding genes, 23 tRNAs, the D-loop region in two fragments and the origin of replication. Manual curation mainly comprises taking and adjusting the rRNA, origin of replication and D-loop region annotations from MITOS2. The both fragments of the D-loop region were merged into one. Annotations of protein coding genes were adjusted from GeSeq and tRNA annotations were taken from tRNAscan.

Assembly finalization. Of all contigs 8 were identified as mitochondrial origin, of which 1 has smaller identity than 90% (minimum 87.5%) to the NOVOplasty mt genome assembly. The removed 8 contigs cover 94.9% of all positions of the NOVOplasty mt genome assembly. After removing contigs of mitochondrial origin, the final assembly contains 1,446,664 contigs with a total length of 1,829,122,027bp, an N50 of 1,468 and an GC of 41.07%.

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