SUPPLEMENTARY MATERIALS

Supplementary Methods

Laboratory methods. For molecular phylogenetic analyses, we extracted total genomic DNA from ethanol-preserved femoral muscle tissue using standard phenol-chloroform-proteinase K extraction with consequent isopropanol precipitation to a final concentration of ~1 mg/mL (protocols followed Hillis et al., 1996 and Sambrook & Russell, 2001). We visualized the isolated total genomic DNA in agarose electrophoresis in the presence of ethidium bromide. We measured the concentration of total DNA in 1 µL using a NanoDrop 2000 (Thermo Scientific, USA), and adjusted the concentration to ca. 100 ng DNA/µL.

We amplified mitochondrial DNA (mtDNA) fragments covering partial sequences of the 16S rRNA mtDNA gene to obtain a 1 088 bp length continuous fragment of mtDNA. This gene is widely applied in biodiversity surveys in amphibians (Vences et al., 2005a, 2005b; Vieites et al., 2009), and has been used in most recent phylogenetic studies on Microhylinae (Matsui et al., 2011; Peloso et al., 2016; Tu et al., 2018) including the genus *Micryletta* (e.g., Poyarkov et al., 2018; Alhadi et al., 2019; Das et al., 2019; Suwannapoom et al., 2020). We performed DNA amplification in 20 µL reactions using 50 ng of genomic DNA, 10 nmol of each primer, 15 nnol of each dNTP, 50 nn`ol additional $MgCl_2$, Taq PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.1 mmol/L $MgCl_2$, and 0.01% gelatin), and 1 unit of Taq DNA polymerase. Primers used in PCR and sequencing included L-2188 (5'-AAAGTGGGCCTAAAAGCAGCCA-3') (Matsui et al., 2006), 16sL1 (5'-CTGACCGTGCAAAGGTAGCGTAATCACT-3') (Hedges, 1994) and 16H-1 (5'-CTCCGGTCTGAACTCAGATCACGTAGG-3') (Hedges, 1994). The polymerase chain reaction (PCR) conditions included an initial denaturation step of 5 min at 94 °C and 43 cycles of denaturation for 1 min at 94 °C, primer annealing for 1 min with the TouchDown program from 65 °C to 55 °C reducing 1 °C every cycle, extension for 1 min at 72 °C, and final extension step for 5 min at 72 °C. The PCR products were loaded onto 1.5% agarose gels in the presence of ethidium bromide and visualized via agarose electrophoresis. When distinct bands were produced, we purified the PCR products using 2 µL of a 1:4 dilution of ExoSapIt (Amersham, USA) per 5 µL of PCR product prior to cycle sequencing. The 10 µL sequencing reaction included 2 µL of template, 2.5 µL of sequencing buffer, 0.8 µL of 10 pmol primer, 0.4 µL of BigDye Terminator v3.1 Sequencing Standard (Applied Biosystems, USA), and 4.2 µL of water. The cycle sequencing used 35 cycles of 10 s at 96 °C, 10 s at 50 °C, and 4 min at 60 °C. We purified the cycle sequencing products by ethanol precipitation. We carried out sequence data collection and visualization on an ABI 3730xl Automated Sequencer (Applied Biosystems, USA). The obtained sequences were deposited in

GenBank under accession numbers MW376732–MW376737 (see Supplementary Table S1).

Phylogenetic analyses. To reconstruct the matrilineal genealogy, we used 16S rRNA sequences of the *Micryletta* sp. from Hainan Island, as well as 16S rRNA sequences of all eight nominal *Micryletta* taxa, including type specimens of *M. aishani* (India), *M. dissimulans* (Songkhla, Thailand), *M. nigromaculata* (Vietnam), and *M. sumatrana* (Sumatra, Indonesia), topotype specimens of *M. inornata* (Sumatra, Indonesia), *M. erythropoda* (Ma Da, Dong Nai, Vietnam), and *M. steinegeri* (Taiwan, China), as well as *M.* cf. *lineata* from southern Thailand and Myanmar (Alhadi et al., 2019; Das et al., 2019; Munir et al., 2020; Poyarkov et al., 2018; Suwannapoom et al., 2020). Unrelated and undescribed lineages of the genus reported in earlier phylogenetic studies were excluded from analysis to avoid confusion and maintain simplicity. We also added a sequence of *Mysticellus franki*, the sister taxon of *Micryletta*, as an outgroup (Garg & Biju, 2019); sequences of *Kaloula pulchra* and *Uperodon systoma* were used to root the tree. GenBank accession numbers, museum vouchers, and origin localities for sequences used in this study are summarized in Supplementary Table S1.

The nucleotide sequences were initially aligned in MAFFT v.6 (Katoh et al., 2002) with default parameters, then manually optimized in BioEdit 7.0.5.2 (Hall, 1999). The uncorrected genetic distances (*p*-distances) between sequences were determined with MEGA 6.0 (Tamura et al., 2013).

We inferred the matrilineal genealogy using Bayesian inference (BI) and maximum-likelihood (ML) approaches. The ML analysis was implemented using the IQ-TREE webserver (Nguyen et al., 2015; Trifinopoulos et al., 2016) preceded by selection of substitution models using the Bayesian Information Criterion (BIC) in ModelFinder (Kalyaanamoorthy et al., 2017) which suggested the GTR+I+G as the best-fitting model of DNA evolution for the 16S rRNA gene fragment. In total, 1 000 bootstrap pseudoreplicates (ML BS) were employed, and nodes with ML BS values ≥90 were considered strongly supported (Felsenstein, 1985), while nodes with values of 75–90 were regarded as significantly supported. Lower values indicated unresolved nodes (Huelsenbeck & Hillis, 1993).

We conducted BI in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003); Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analyses were run with one cold chain and three heated chains for one million generations, with sampling every 100 generations. We performed five independent MCMCMC runs and the initial 2 500 trees were discarded as burn-in. We assessed confidence in tree topology by Bayesian posterior probability (BPP) (Huelsenbeck & Ronquist, 2001). Nodes with BPP≥0.95 were considered strongly supported (Huelsenbeck & Ronquist, 2001; Wilcox et al., 2002), while nodes with values of 0.90–0.94 were considered well supported.

Morphology. The measurements and descriptions of morphological characteristics

followed Fei et al. (2009) and Poyarkov et al. (2018). Measurements were taken to the nearest 0.1 mm with digital calipers: snout-vent length (SVL); head length from tip of snout to hind border of jaw angle (HL); head width at commissure of jaws (HW); snout length from tip of snout to anterior corner of eye (SL); eye diameter measured as distance between anterior and posterior corners of eye (EL); nostril-eyelid length measured as distance between anterior corner of eye and nostril center (N-EL); internasal distance (IND); interorbital distance (IOD); upper eyelid width measured as greatest width of upper eyelid (UEW); horizontal diameter of tympanum (TMP); forelimb length measured as length of straightened forelimb from arm insertion to tip of third finger (FLL); lower arm and hand length measured as distance from elbow to tip of third finger (LAHL); first finger length measured from distal end of inner palmar tubercle to tip of first finger (1FL); hand length from tip of third finger to proximal edge of outer palmar tubercle (HAL); hindlimb length measured as length of straightened hindlimb from groin to tip of fourth toe (HLL); tibia length with hindlimb flexed (TL); foot length from tip of fourth toe to proximal edge of inner metatarsal tubercle (FL); inner metatarsal tubercle length measured as maximal diameter of inner metatarsal tubercle (IMTL); first toe length measured from distal end of inner metatarsal tubercle to tip of first toe (1TOEL). Webbing formulae are given following Savage (1975). Sex was determined by direct observation of calling in life and the presence of internal vocal sac openings or of eggs in the abdomen.

Due to the high likelihood of undiagnosed diversity within the genus, where available, we relied on examination of original species descriptions and topotypic materials for comparative morphological data of all eight named taxa of the genus *Micryletta*: *M. aishani* (Das et al., 2019); *M. dissimulans* (Suwannapoom et al., 2020) *M. erythropoda* (Tarkhnishvili, 1994; Vassilieva et al., 2016), *M. inornata* (Boulenger, 1890; Alhadi et al., 2019), *M. lineata* (Taylor, 1962), *M. nigromaculata* (Poyarkov et al., 2018), *M. steinegeri* (Boulenger, 1909; Das et al., 2019; Fei et al., 2009, 2012), and *M. sumatrana* (Munir et al., 2020).

Acoustic analyses. The advertisement call was recorded in the field using a TASCAM DR-40 digital sound recorder (44.1 kHz sampling rate and 24 bit encoding) held within 0.5 m of the calling individual. Calls were analysed with Raven Pro v.1.5 software (Cornell Lab of Ornithology, available from http://www.birds.cornell.edu/raven). The spectrograms and waveforms were generated by Raven Pro 1.5 software with fast Fourier transform of 512 points and 50% overlap using the Hanning window function, from which all parameters and characters were measured. For each call recording, we measured call duration (s), number of pulses per note, intercall-interval (s), call repetition rate (calls/s), and peak frequency (kHz) following the terminology of Köhler et al. (2017). Ambient air temperature of the calling site was taken with a digital thermometer (Model: Peakmeter MS6508).

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Supplementary Tables

Supplementary Table S1 Localities, voucher information, and GenBank accession numbers for all specimens used in molecular analyses in this study

Species	Specimen ID	Locality	GenBank accession No.	Reference
M. aishani	SDBDU 3920	India: Assam, Cachar district, Subhong (1)	MK889218	Das et al. (2019)
M. dissimulans	AUP01690	Thailand: Songkla Prov., Saba Yoi district (14)	MT573414	Suwannapoom et al. (2020)
M. dissimulans	AUP01691	Thailand: Songkla Prov., Saba Yoi district (14)	MT573415	Suwannapoom et al. (2020)
M. dissimulans	AUP01696	Thailand: Songkla Prov., Saba Yoi district (14)	MT573416	Suwannapoom et al. (2020)
M. dissimulans	AUP01698	Thailand: Songkla Prov., Saba Yoi district (14)	MT573413	Suwannapoom et al. (2020)
M. erythropoda	ZMMU A4721-1533	Vietnam: Dong Nai, Ma Da (Vinh Cuu) N.R. (10)	MH756146	Poyarkov et al. (2018)
M. erythropoda	ZMMU A4721-1542	Vietnam: Dong Nai, Ma Da (Vinh Cuu) N.R. (10)	MH756147	Poyarkov et al. (2018)
M. immaculata **sp. nov.**	KFBG 14270	China: Hainan: Exian (5)	MW376736	*this paper*
M. immaculata **sp. nov.**	KFBG 14271	China: Hainan: Exian (5)	MW376737	*this paper*
M. inornata	MZB Amph 23949	Indonesia: Sumatra, Deli Serdang (16)	LC208135	Alhadi et al. (2019)
M. inornata	MZB Amph 23947	Indonesia: Sumatra, Deli Serdang (16)	LC208136	Alhadi et al. (2019)
M. inornata	MZB Amph 23948	Indonesia: Sumatra, Deli Serdang (16)	LC208137	Alhadi et al. (2019)
M. inornata	MZB Amph 27242	Indonesia: Sumatra, Aceh (15)	LC208138	Alhadi et al. (2019)
M. cf. *lineata*	KUHE 23858	Thailand: Ranong (12)	AB634695	Matsui et al. (2011)
M. cf. *lineata*	CAS 247206	Myanmar: Tanintharyi Div., Kawthaung dist. (11)	KM509167	Peloso et al. (2016)
M. nigromaculata	ZMMU A5947	Vietnam: Hai Phong, Cat Ba N.P. (3)	MH756148	Poyarkov et al. (2018)
M. nigromaculata	ZMMU A5937	Vietnam: Hai Phong, Cat Ba N.P. (3)	MH756149	Poyarkov et al. (2018)
M. nigromaculata	ZMMU A5946	Vietnam: Hai Phong, Cat Ba N.P. (3)	MH756151	Poyarkov et al. (2018)
M. nigromaculata	DTU 301	Vietnam: Ninh Binh, Cuc	MH756154	Poyarkov et al. (2018)

		Phuong N.P. (2)		
M. steinegeri	KUHE 35937	China: Taiwan: Yunlin (9)	AB634696	Matsui et al. (2011)
M. steinegeri	ZMMU A5336-1	China: Taiwan: Kaohsiung (7)	MW376732	*this paper*
M. steinegeri	ZMMU A5336-2	China: Taiwan: Kaohsiung (7)	MW376733	*this paper*
M. steinegeri	ZMMU A5336-3	China: Taiwan: Kaohsiung (7)	MW376734	*this paper*
M. steinegeri	released	China: Taiwan: Tainan (8)	MW376735	*this paper*
M. sumatrana		Indonesia,	MN727065	Munir et al. (2020)
Outgroup				
Mysticellus franki	ZSI/WGRC/V/A/967	India: Kerala, Wayand	MK285340	Garg and Biju (2019)
Uperodon systoma	SDBDU 2005.4723	India: Tamil Nadu: Kunnapattu	MG557949	Garg et al. (2019)
Kaloula pulchra	NMNS 3208	China	KC822614	Blackburn et al. (2013)

Locality numbers correspond to those in Figure 1. For references see Supplementary Methods section.

Supplementary Table S2 Uncorrected *p*-distances (percentage) of 16S rRNA sequences of *Micryletta* species included in phylogenetic analyses (below diagonal), average intraspecific genetic *p*-distances (on diagonal, in bold), and standard error estimates (above diagonal)

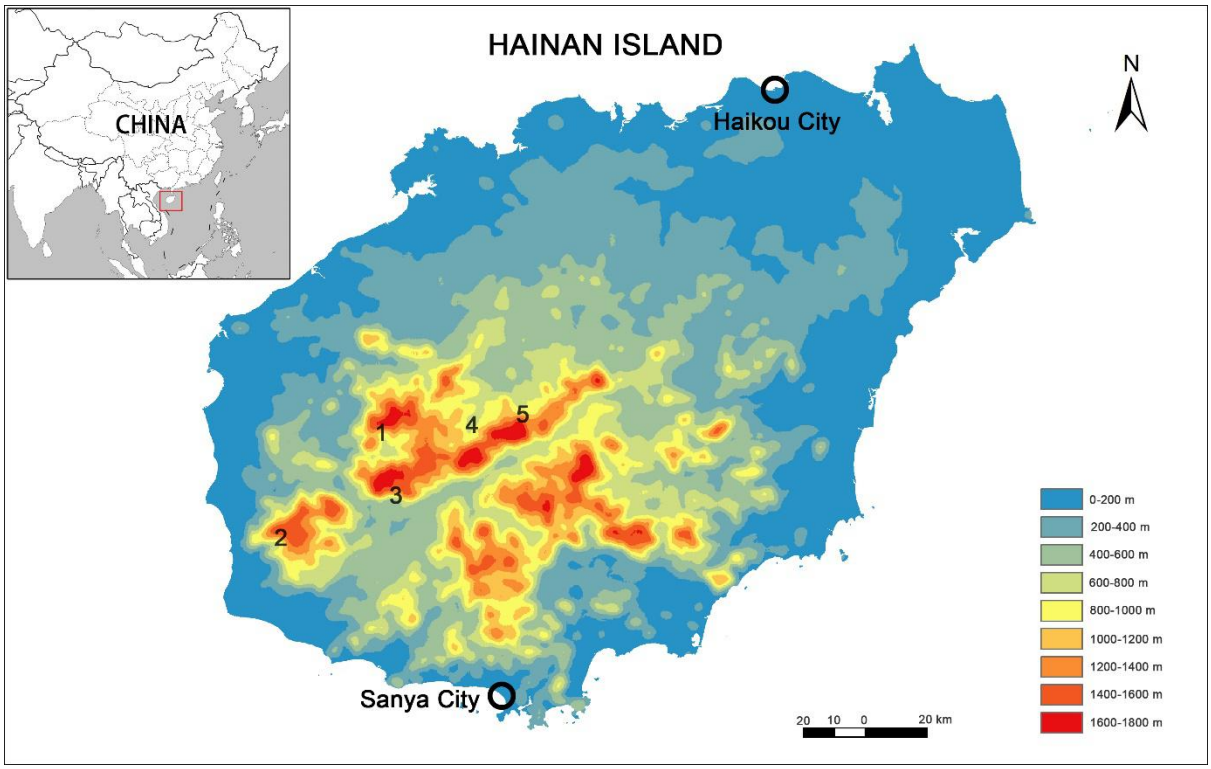
	Species	1	2	3	4	5	6	7	8	9	10
1	*M. immaculata* **sp. nov.**	**0.0**	0.7	1.1	1.1	1.1	1.0	1.1	1.2	1.2	1.3
2	*M. steinegeri*	3.0	**0.1**	1.0	0.9	1.0	0.9	1.0	1.1	1.0	1.3
3	*M. inornata*	6.7	5.0	**0.6**	1.0	1.2	1.1	1.1	1.1	1.2	1.3
4	*M. aishani*	4.8	3.5	4.7	—	1.0	0.8	0.9	0.9	1.0	1.4
5	*M. erythropoda*	6.8	5.5	7.1	4.8	**0.0**	0.7	1.1	1.2	1.3	1.4
6	*M. cf. lineata*	5.6	4.1	6.1	3.4	2.6	**0.0**	1.0	1.1	1.1	1.4
7	*M. nigromaculata*	6.9	5.2	5.9	4.7	7.7	5.6	**0.7**	1.0	1.0	1.5
8	*M. dissimulans*	6.4	4.8	5.5	4.4	7.4	6.0	5.0	**0.0**	1.1	1.3
9	*M. sumatrana*	7.7	6.0	7.8	6.0	9.2	7.5	5.9	5.1	—	1.5
10	*Mysticellus*	9.5	8.8	9.5	9.0	9.7	8.8	9.6	9.0	10.7	—

Supplementary Table S3 Morphological comparison of *Micryletta* species in key morphological characters. Tables in gray indicate distinct differences when compared to *Micryletta immaculata* **sp. nov.**

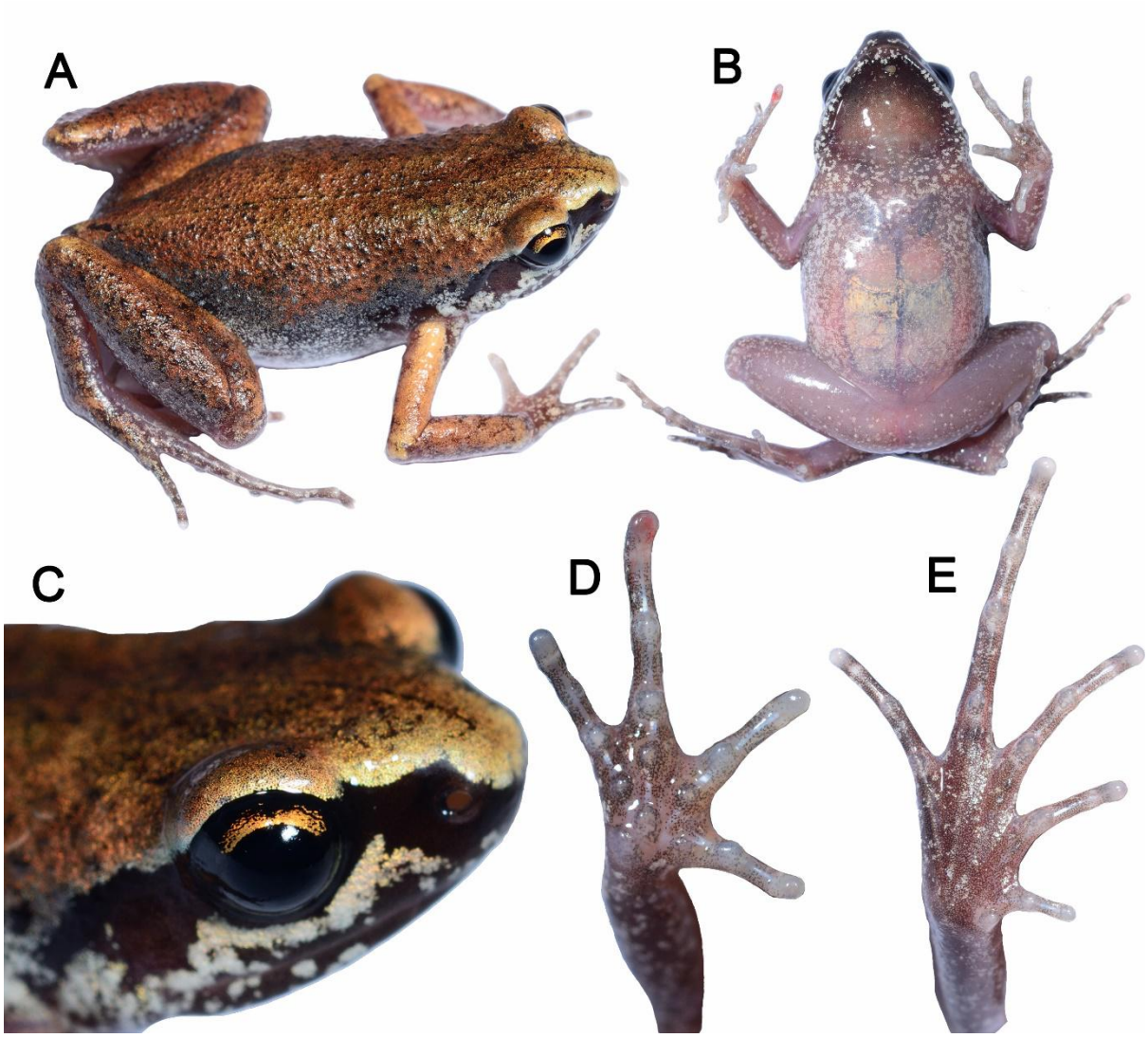
	Male SVL (mm)	Female SVL (mm)	Supratympanic fold	White spots/line on the upper lip	Dorsum coloration in life	Dark patterns on dorsum	Dark patterns on flanks	Toe webbing	Where on body tibiotarsal articulation reaches at adpressed limb	outer metatarsal tubercle	References
M. immaculata **sp. nov.**	23.3–24.8 (*n*=3)	27.7–30.1 (*n*=2)	distinct	present	bronze brown to reddish brown	absent	absent	basal and poorly developed	tympanum	absent	This study
M. aishani	22.1–23.5 (*n*=7)	25.6–27.3 (*n*=4)	weakly-developed	present	brown to reddish-brown	few scattered blackish-brown spots on posterior parts of the back and near the groin	a prominent blackish-brown streak	absent	armpit	absent	Das et al., 2019
M. inornata	16.8–20.5 (*n*=3)	19.5 (*n*=1)	weakly-developed	present	brownish-grey	irregular blackish-brown blotches and a discontinuous lateral blackish-brown streak	blackish-brown spots	absent	eye	absent	Boulenger, 1890; Alhadi et al., 2019
M. lineata	19.0–19.2 (*n*=2)	19.2–22 (*n*=2)	distinct	present	brownish-grey	three straight continuous or broken lines	a black stripe	absent	eye	absent	Taylor, 1962
M. dissimulans	20.3–22.4 (*n*=7)	24.4–26.7 (*n*=2)	indistinct and flat	absent	reddish-brown	irregular-shaped brown blotches edged in beige	large black spots	absent	tympanum	absent	Suwannapoom et al., 2020

M. erythropoda	up to 30 mm	up to 27 mm	distinct	present	gray or beige to saturated ochre or brick-red	large and distinct dark brown spots/strips	large and distinct dark brown spots/strips	absent	posterior edge of tympanum	present	Tarkhnishvili, 1994; Vassilieva et al., 2016; Poyarkov et al., 2018
M. nigromaculata	18.5–23.0 (*n*=18)	24.2–25.9 (*n*=3)	distinct and thick	absent	brown to reddish-brown	irregular dark-brown hourglass-shaped pattern and two large dark inguinal spots	dark patches or spots	absent	eye	absent	Poyarkov et al., 2018
M. sumatrana	17.4 (*n*=1)	22.8 (*n*=1)	distinct and thick	present	golden brown	Dorsum scattered with black spots; an inguinal blackish blotch present	irregular blackish patches with cream mottling	absent	front of eye	absent	Munir et al., 2020
M. steinegeri	up to 24.3 mm	up to 30 mm	weak and indistinct	present	dark grey to violet	irregular dark blotches or speckles	a dark streak or irregular dark speckles	rudimentary webbing	tympanum	absent	Boulenger, 1909; Das et al., 2019; Fei et al., 2009

Supplementary Figures

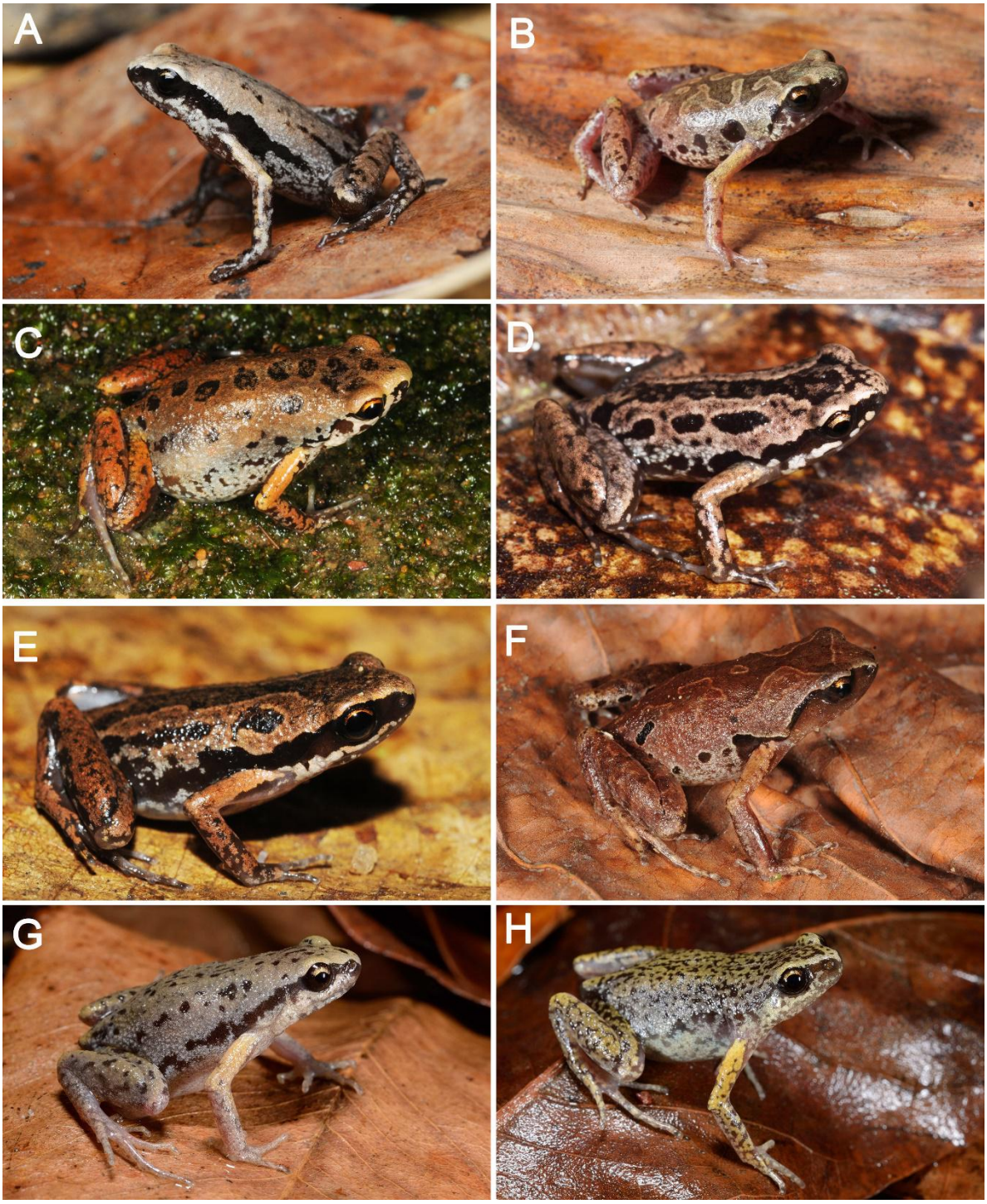


Supplementary Figure S1. Distribution of *Micryletta immaculata* **sp. nov.** on Hainan Island, China. 1: Exianling Nature Reserve, Dongfang City (locality of holotype); 2: Jianfengling National Forest Park, Ledong County; 3: Baoxiu Village, Jiaxi Nature Reserve, Ledong County; 4: Yaxing Village, Yinggeling National Nature Reserve, Baisha County; 5: Zhufeng of Yinggezui substation, Yinggeling National Nature Reserve, Baisha County.



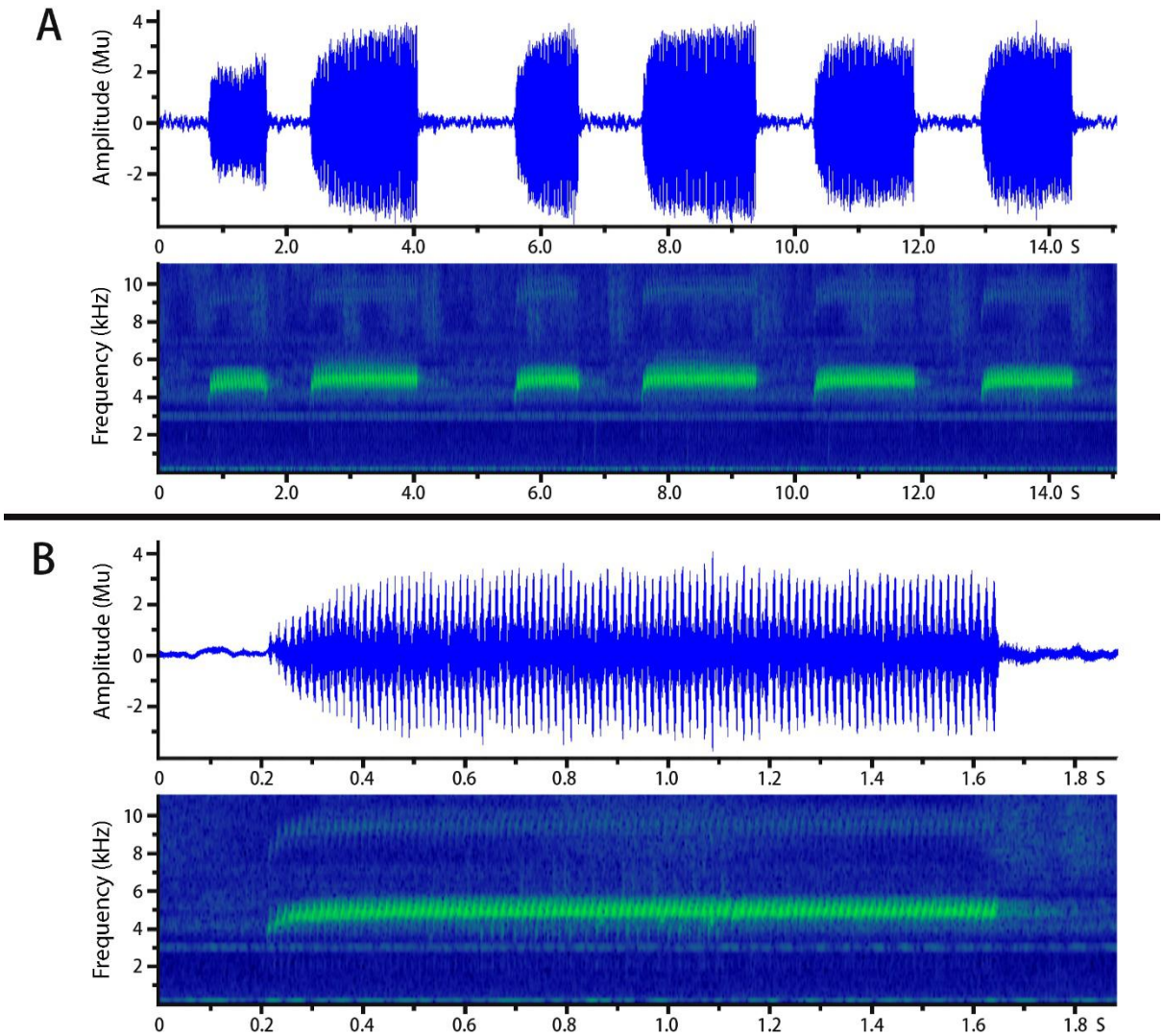
Supplementary Figure S2. Holotype of *Micryletta immaculata* **sp. nov.** (KFBG 14271) in life. A: dorsolateral view; B: ventral view; C: lateral view of head, note coloration of iris; D: ventral view of right hand; E: ventral view of right foot. Photos by J.H. Yang.

Supplementary Figure S3. *Micryletta immaculata* **sp. nov.** in life. A: KFBG 14271, adult male holotype; B: KFBG 14105, adult female paratype; C: KFBG 14272, adult male paratype with blind right eye; D: adult male from Yaxing Village, Yinggeling National Nature Reserve, photographed on 29 August 2012 (not collected); E–F: KFBG 14106, gravid female paratype. Photos by J.H. Yang (A, C–F) and Jay Wan (B).



Supplementary Figure S4. A: *M. aishani* from Indawgyi, Kachin, Myanmar; **B:** Holotype of *M. dissimulans* from Songkhla, Thailand; C: *M. erythropoda* sensu stricto from Ma Da, Dong Nai Province, southern Vietnam (type locality); D: *M. inornata* sensu stricto from Sumatra, Indonesia; E: *M.* cf. *lineata* from Suratthani, Thailand (close to type locality of *M. inornata lineata* in Nakhon Si Thammarat, ca. 95 km distance); F: Holotype of *M. nigromaculata* from Hai Phong, Vietnam; G–H: *M. steinegeri* sensu stricto from Tainan of Taiwan Island. Photos by N.A. Poyarkov (A, C, E, F), P. Pawangkhanant (B), J.H. Yang (D), and Chung-Wei You (G–H).

Supplementary Figure S5. Breeding microhabitats of *Micryletta immaculata* **sp. nov.** in Hainan. A: breeding water puddle on surface of limestone rock in Exianling Nature Reserve, green circle shows calling male holotype KFBG 14271; B: breeding pond in Zhufeng, Yinggeling National Nature Reserve on 31 May 2005, inserted small photo shows pair in amplexus. Photos by J.H. Yang (A) and Bosco P.L. Chan (B).



Supplementary Figure S6. Advertisement call of male *Micryletta immaculata* **sp. nov**. (paratype KFBG 14270). A: waveform (Mu) and spectrogram (kHz) showing six-call portion of a call series; B: waveform and spectrogram showing a single call.