Supplementary Materials

Supplementary Methods

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

We amplified mtDNA fragments covering partial sequences of the 16S rRNA mtDNA gene to obtain a 1 036 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 (Gorin et al., 2021; Matsui et al., 2011; Peloso et al., 2016; Tu et al., 2018), including the genus Micryletta (e.g., Alhadi et al., 2019; Das et al., 2019; Miller et al., 2021; Poyarkov et al., 2018; Suwannapoom et al., 2020; Yang & Poyarkov, 2021). We performed DNA amplification in 20 µL reactions using ca. 50 ng of genomic DNA, 10 nmol of each primer, 15 nMol of each dNTP, 50 nMol additional MgCl₂, Taq PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.1 mmol/L MgCl₂, and 0.01% gelatin), and 1 unit of Taq DNA polymerase. Primers used for polymerase chain reaction (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 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% 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. Cycle sequencing consisted of 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 MZ474684–MZ474685 (see Supplementary Table S1).

Phylogenetic analyses. To reconstruct the matrilineal genealogy, we used the 16S rRNA sequences of *Micryletta* sp. from Lam Dong Province, as well as the 16S rRNA sequences of all ten nominal *Micryletta* taxa, including the type specimens of *M. aishani* (India), *M. dissimulans* (Songkhla, Thailand), *M. hekouensis* (China), *M. nigromaculata* (Vietnam), *M. sumatrana* (Sumatra, Indonesia), and *M. immaculata* (Hainan, China), and 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; Liu et al., 2021; Munir et al., 2020; Poyarkov et al., 2018; Suwannapoom et al., 2020; Yang & Poyarkov, 2021). We also added a sequence of *Mysticellus franki* Garg & Biju, the sister taxon of *Micryletta*, as an outgroup (Garg & Biju, 2019);

sequences of *Kaloula pulchra* Gray and *Uperodon systoma* (Schneider) 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.

We initially aligned nucleotide sequences using ClustalX v1.81 (Thompson et al., 1997) with default parameters, and then optimized them manually in BioEdit v7.0.5.2 (Hall, 1999) and MEGA v6.0 (Tamura et al., 2013). We used MODELTEST v.3.06 (Posada & Crandall, 1998) to estimate the optimal evolutionary models to be used for dataset analysis. The best-fitting model for the 16S rRNA gene fragment was the GTR+G model of DNA evolution, as suggested by the Akaike Information Criterion (AIC). We determined mean uncorrected genetic distances (*p*-distances) between sequences with MEGA v6.0 (Supplementary Table S2).

We inferred the matrilineal genealogy using Bayesian inference (BI) and maximumlikelihood (ML) approaches. We conducted BI using MrBayes v3.1.2 (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 and sampling every 1 000 generations. We performed two independent MCMCMC runs, with the first 200 trees discarded as burn-in. We assessed confidence in tree topology by the frequency of nodal resolution (posterior probability; BI PP) (Huelsenbeck & Ronquist, 2001). We used IQ-TREE v1.6.12 (Nguyen et al., 2015) to reconstruct ML phylogenies. Confidence in tree topology for ML analysis was assessed by 10 000 ultrafast bootstrap replications for ML analysis (UFB) (Minh et al., 2013). In both datasets, we regarded tree nodes with BI PP and UFB values over 0.95 to be sufficiently resolved *a priori*. BI PP and UFB values between 0.95 and 0.90 were regarded as tendencies. Lower values indicated unresolved nodes (Huelsenbeck & Hillis, 1993; Minh et al., 2013).

Morphology. Measurements were taken using a digital caliper under a light dissecting microscope to the nearest 0.01 mm, subsequently rounded to 0.1 mm. Morphometrics of adults and character terminology followed Poyarkov et al. (2018): (1) snout-vent length (SVL; tip of snout to cloaca); (2) head length (HL; tip of snout to hind border of jaw angle); (3) snout length (SL; anterior corner of eye to tip of snout); (4) eye length (EL; distance between anterior and posterior corners of eye); (5) nostril-eye length (N-EL; distance between anterior corner of eye and nostril center); (6) head width (HW; maximum width of head at level of mouth angles in ventral view); (7) internarial distance (IND; distance between central points of nostrils); (8) interorbital distance (IOD; shortest distance between medial edges of eyeballs in dorsal view); (9) upper eyelid width (UEW; maximum distance between medial edge of eyeball and lateral edge of upper eyelid); (10) tympanum length (TMP, horizontal tympanum diameter); (11) forelimb length (FLL; length of straightened forelimb to tip of third finger); (12) lower arm and hand length (LAL; distance between elbow and tip of third finger); (13) hand length (HAL; distance between proximal end of outer palmar (metacarpal) tubercle and tip of third finger); (14) first finger length (1FL, distance between tip and distal end of inner palmar tubercle); (15) inner palmar tubercle length (IPTL; maximum distance between proximal and distal ends of inner palmar tubercle); (16) outer palmar tubercle length (OPTL; maximum diameter of outer palmar tubercle); (17) third finger disk diameter (3FDD); (18) hindlimb length (HLL; length of straightened hindlimb from groin to tip of fourth toe); (19) tibia length (TL; distance between knee and tibiotarsal articulation); (20) foot length (FL; distance between distal end of tibia and tip of fourth toe); (21) inner metatarsal tubercle length (IMTL; maximum length of inner metatarsal tubercle); (22) first toe length (1TOEL), distance between distal end of inner metatarsal tubercle and tip of first toe; (23) fourth toe disk diameter (4TDD). Terminology for describing eye coloration in living individuals followed Glaw & Vences (1997); webbing and subarticular tubercle formulas followed those of Savage (1975). All measurements were taken on the right side of the examined specimen. Sex was determined by dissection of the body

cavity and gonadal examination. Other abbreviations. Mt.: mountain; NP.: National Park; a.s.l.: above sea level. We compared morphological characters of the new species with other members of the genus and comparative data obtained from the literature: Alhadi et al., 2019; Boulenger, 1909; Das et al., 2019; Hornell, 1890; Munir et al., 2020; Poyarkov et al., 2018; Suwannapoom et al., 2020; Tarkhnishvili, 1994 (see Supplementary Table S3).

Staging of larvae followed Gosner (1960); Morphometric data and abbreviations for larval characters followed Altig (2007) and included the following parameters: total length (TL); body length (BL); tail length (TaL); tail base width (TB); maximal body width (BW); maximal body height (BH); maximal tail height (MTH); snout-vent length (SVL); snout-spiracle distance (SS); maximal dorsal tail fin height (DF); maximal ventral tail fin height (VF); interpupillary distance (IP); snout-pupillary distance (SP); horizontal eye diameter (ED); and horizontal mouth width (MW).

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Supplementary Tables

Supplementary Table S1. Localities, voucher information, and GenBank accession numbers for specimens used in molecular analyses in this study. Locality numbers correspond to those in Figure 1. For references, see Supplementary Methods section.

Species	Specimen ID	Locality	GenBank accession No.	Reference
M. aishani	SDBDU 3920	India: Assam, Cachar, Subhong (1)	MK889218	Das et al. (2019)
M. aishani	CAS 231526	Myanmar: Kachin, Myitkyina, Moenyin (2)	MW035599	Miller et al. (2021)
M. dissimulans	AUP01690	Thailand: Songkhla, Saba Yoi (18)	MT573414	Suwannapoom et al. (2020)
M. dissimulans	AUP01691	Thailand: Songkhla, Saba Yoi (18)	MT573415	Suwannapoom et al. (2020)
M. dissimulans	AUP01696	Thailand: Songkhla, Saba Yoi (18)	MT573416	Suwannapoom et al. (2020)
M. dissimulans	AUP01698	Thailand: Songkhla, Saba Yoi (18)	MT573413	Suwannapoom et al. (2020)
M. erythropoda	ZMMU A4721-1533	Vietnam: Dong Nai, Ma Da (Vinh Cuu) N.R. (11)	MH756146	Poyarkov et al. (2018)
M. erythropoda	ZMMU A4721-1542	Vietnam: Dong Nai, Ma Da (Vinh Cuu) N.R. (11)	MH756147	Poyarkov et al. (2018)
M. hekouensis	KIZ 20210510	China: Yunnan, Hekou (3)	MZ536627	Liu et al. (2021)
M. hekouensis	KIZ 20210511	China: Yunnan, Hekou (3)	MZ536628	Liu et al. (2021)
M. immaculata	KFBG 14270	China: Hainan: Exian (6)	MW376736	Yang & Poyarkov (2021)
M. immaculata	KFBG 14271	China: Hainan: Exian (6)	MW376737	Yang & Poyarkov (2021)
M. inornata	USNM Herp 587901	Myanmar: Tanintharyi, Ywahilu (15)	MT609034	Miller et al. (2021)
M. inornata	MZB Amph 23949	Indonesia: Sumatra, Deli, Serdang (17)	LC208135	Alhadi et al. (2019)
M. inornata	MZB Amph 23947	Indonesia: Sumatra, Deli, Serdang (17)	LC208136	Alhadi et al. (2019)
M. inornata	MZB Amph 23948	Indonesia: Sumatra, Deli, Serdang (17)	LC208137	Alhadi et al. (2019)
M. inornata	MZB Amph 27242	Indonesia: Sumatra, Aceh (16)	LC208138	Alhadi et al. (2019)
<i>M.</i> cf. <i>lineata</i>	KUHE 23858	Thailand: Ranong (14)	AB634695	Matsui et al. (2011)
<i>M</i> . cf. <i>lineata</i>	CAS 247206	Myanmar: Tanintharyi, Kawthaung (13)	KM509167	Peloso et al. (2016)
M. cf. lineata	CAS247199	Myanmar: Tanintharyi, Kawthaung, Khamaukgyi (12)	MW042900	Miller et al. (2021)
Micryletta melanops sp. nov.	ZMMU NAP-01381- 5	Vietnam: Lam Dong: Bidoup–Nui Ba N.P. (10)	MZ474685	This paper

Micryletta melanops sp. nov.	ZMMU A7583	Vietnam: Lam Dong: Bidoup–Nui Ba N.P. (10)	MZ474684	This paper
M. nigromaculata	ZMMU A5947	Vietnam: Hai Phong, Cat Ba N.P. (5)	MH756148	Poyarkov et al. (2018)
M. nigromaculata	ZMMU A5937	Vietnam: Hai Phong, Cat Ba N.P. (5)	MH756149	Poyarkov et al. (2018)
M. nigromaculata	ZMMU A5946	Vietnam: Hai Phong, Cat Ba N.P. (5)	MH756151	Poyarkov et al. (2018)
M. nigromaculata	DTU 301	Vietnam: Ninh Binh, Cuc Phuong N.P. (4)	MH756154	Poyarkov et al. (2018)
M. steinegeri	KUHE 35937	China: Taiwan: Yunlin (9)	AB634696	Matsui et al. (2011)
M. steinegeri	ZMMU A5336-1	China: Taiwan: Kaohsiung (7)	MW376732	Yang & Poyarkov (2021)
M. steinegeri	ZMMU A5336-2	China: Taiwan: Kaohsiung (7)	MW376733	Yang & Poyarkov (2021)
M. steinegeri	ZMMU A5336-3	China: Taiwan: Kaohsiung (7)	MW376734	Yang & Poyarkov (2021)
M. steinegeri	released	China: Taiwan: Tainan (8)	MW376735	Yang & Poyarkov (2021)
M. sumatrana	MZB Amph 30594	Indonesia: Sumatra: Sako Suban (19)	MN727065	Munir et al. (2020)
Outgroup				
Mysticellus franki	ZSI/WGRC/V/A/967	India: Kerala, Wayand	MK285340	Garg & 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)

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	11
1	M. melanops sp. nov.	0.0	0.7	0.9	1.0	0.7	1.0	1.0	0.9	0.9	0.8	1.1
2	M. aishani	2.6	0.1	0.9	1.0	0.7	1.1	1.0	0.8	0.9	0.9	1.0
3	M. dissimulans	4.4	4.4	0.0	1.2	0.9	1.2	1.1	1.1	1.0	1.1	1.1
4	M. erythropoda	5.0	4.8	7.4	0.0	1.0	0.7	1.2	0.7	1.1	1.0	1.3
5	M. hekouensis	3.0	3.2	5.0	5.0	0.0	0.7	1.0	0.8	1.0	0.5	1.1
6	M. immaculata	5.0	4.8	6.4	6.8	3.2	0.0	1.1	1.0	1.1	0.7	1.2
7	M. inornata	4.9	4.7	5.5	7.1	5.3	6.7	1.2	1.1	1.1	1.0	1.2
8	M. cf. lineata	4.0	3.4	6.0	2.6	3.6	5.6	6.1	0.3	1.0	0.9	1.1
9	M. nigromaculata	4.7	4.7	5.0	7.7	5.6	6.9	5.9	7.7	0.7	1.0	1.0
10	M. steinegeri	3.7	3.5	4.8	5.5	1.2	2.9	5.0	4.1	5.2	0.1	1.0
11	M. sumatrana	5.8	6.0	5.1	9.2	6.6	7.7	7.8	7.5	5.9	6.0	—

Supplementary Table S3. Morphological comparison of *Micryletta melanops* sp. nov with 10 currently recognized species of the genus *Micryletta* (? – no data).

Character	M. melanops sp. nov	M. aishani	M. dissmulans	M. erythropoda	M. hekouensis	M. inornata
SVL (Males, mm)	?	22.1–23.5	24.4–26.7	up to 30.0	20.5	16.8–20.5
SVL (Females, mm)	22.4	25.6–27.3	20.3-22.4	?	20.8	19.5
Iris color	uniform black	bicolored	bicolored	bicolored	bicolored	bicolored
		(upper third light brown)	(upper third golden)	(upper third golden)	(upper third bronze)	(upper third golden)
Snout in dorsal view	truncate	nearly truncate	rounded	obtuse	abruptly rounded	nearly rounded
Foot webbing	rudimentary	absent	absent	rudimentary	absent	absent
Outer metatarsal tubercle	absent	absent	absent	present	absent	absent
Tibiotarsal articulation of adpressed limb reaching up to	the level of eye	the level of armpits	the level of tympanum	the level posterior edge of tympanum	the level front of the eye	the level of eye
Supratympanic fold	distinct	weakly-developed	distinct	indistinct	very indistinct	rather indistinct
Dorsal skin texture	smooth	shagreened with minute spinules	slightly granulated to shagreened	smooth	smooth	smooth with small tubercles
Dorsal coloration	orange red	brown to reddish- brown	reddish-brown	grey or beige to saturated ochre or brick-red	golden-brown	brownish-grey with a silver tinge

Dorsal color pattern	small brown patches forming reticulate pattern	with faint median band	with confluent brown blotches edged with beige	irregular dark spots	brownish-black stripes and blotches	irregular blackish- brown blotches
Coloration of lateral sides of the head	immaculate dark brown without white patches	blackish-brown with white spots	dark-brown to black lacking white patches along the upper lip	dark brown with white spots	dark brown with white spots	blackish-brown with silver white line along upper lip
Coloration of flanks	dark brown anteriorly fading posteriorly with brown spots in the groin area	prominent dark blackish-brown streak from tip of the snout up to the lower abdomen on either side	brown with dark spots and whitish mottling	dark brown to grey with white patches	longitudinal dark- gray stripe	light brown with blackish brown spots
Ventral coloration	uniform dark-grey	ash-grey with a purplish tinge and brown mottling towards the margins	pinkish to bluish-grey laterally with brown marbled pattern	light grey with mottling	pinkish-brown with white marbling on chest and lateral sides of belly	light reddish-grey without mottling
Distribution	southern Vietnam (Lam Dong)	northeast India (Assam, Mizoram), Bangladesh (Sylhet)	southern Thailand (Songkhla)	southern Vietnam (Kon Tum, Gia Lai, Dak Lak, Dak Nong, Binh Phuoc, Ba Ria- Vung Tau, Tay Ninh, Dong Nai, An Giang, Kien Giang)	southern China (Yunnan Province)	Indonesia (northern Sumatra)
Source	our data	Das et al., 2019	Suwannapoom et al., 2020:	Tarkhnishvili, 1994; our data	Liu et al., 2021	Alhadi et al., 2019;
			our data			Das et al., 2019; our data

Continued

Character	M. immaculata	M. nigromaculata	M. steinegeri	M. sumatrana	M. lineata	M. cf. lineata
SVL (Males, mm)	23.3–24.8	18.5–23.3	23.0-23.5	17.4	19.0–19.2	22.1–23.5
SVL (Females, mm)	27.7–30.1	24.2–25.9	27.0-30.1	22.8	19.2–22.0	21.6–28.3
Iris color	bicolored (upper	bicolored	bicolored	bicolored	bicolored	bicolored
	unita bionze)	(upper third	(upper third	(upper third copper-	(upper and lower	(upper third light
		golden)	golden)	orange)	thirds golden)	golden)
Snout ventral view	abruptly rounded	rounded	rounded to truncate	rounded	nearly rounded	slightly truncate
Foot webbing	rudimentary	absent	rudimentary	absent	absent	absent
Outer metatarsal tubercle	absent	absent	absent	absent	absent	absent
Tibiotarsal articulation of	the level of	the level of eye	the level of	the level of eye	the level of eye	?
adpressed limb reaching	tympanum		tympanum			
up to						
Supratympanic fold	distinct	distinct	rather indistinct	rather indistinct	distinct	indistinct
Dorsal skin texture	smooth with tiny	slightly granular	smooth to	smooth	smooth	moderately shagreen
	flat tubercles	with small round flattened tubercles	shagreened			
Dorsal coloration	uniform bronze- brown	brown to reddish- brown	dark grey to violet	golden brown	brownish-gray	grayish-brown to rufous brown

Dorsum color pattern	no pattern	hourglass-shaped	irregular dark blotches or speckles	without dark pattern	three straight continuous or broken dark lines	three or four rows of scattered dark marks
Coloration of lateral sides of the head	dark brown with white spots along upper lip	dark-brown without white patches	grey-brown with a series of white spots	cream spots on lips, tympanum region, and axilla present	white stripe or spots from snout to axilla	white stripe or spots from snout to axilla
Coloration of flanks	with silver white spots	brown with dark patches or spots edged with white	grey-brown with dark marbling	dark brown with white patches	dark stripe from snout to groin with indistinct light stripe ventrally	dark stripe from snout to midtrunk
Ventral coloration	irregular white marbling patterns	whitish without mottling	greyish-white with mottling	with dark brown background coloration with cream mottling	light grey without mottling	beige with light- brown mottling
Distribution	southern China (Hainan Island)	northern Vietnam (Hai Phong, Ninh Binh)	eastern China (Taiwan Island)	Indonesia (southern Sumatra)	Peninsular Thailand	Myanmar (Tanintharyi)
Source	Yang & Poyarkov, 2021	Poyarkov et al., 2018; our data	Alhadi et al., 2019; Das et al., 2019; our data	Munir et al., 2020	Taylor, 1962; our data	Zug & Mulcahy, 2020; Miller et al., 2021



Supplementary Figure S1. Holotype of *Micryletta melanops* **sp. nov.** (ZMMU A7583) in preservative. A: dorsal view; B: ventral view; C: lateral view of head. Photos by N.A. Poyarkov.



Supplementary Figure S2. Holotype of *Micryletta melanops* **sp. nov.** (ZMMU A7583). A: ventral view of right hand; B: ventral view of right foot. Drawings by N.A. Poyarkov.



Supplementary Figure S3. Tadpole of *Micryletta melanops* **sp. nov.** A: ZMMUNAP-01381-1 (Gosner stage 34), lateral view; B: drawing showing general morphology of NAP-01381-1 in lateral view; C: drawing showing mouthparts of NAP-01381-1 in dorsal aspect. Photo and drawings by N.A. Poyarkov.



Supplementary Figure S4. Iris coloration in eleven species of the genus *Micryletta*. A – *Micryletta aishani* (Sagaing, Myanmar); B – *M. dissimulans* (Songkhla, Thailand); C – *M. erythropoda* (Dak Lak, Vietnam); D – *M. hekouensis* (Yunnan, China); E – *M. immaculata* (Hainan, China); F – *M. inornata* (North Sumatra, Indonesia); G – *M. lineata* (Nakhon Si Thammarat, Thailand); H – *M. nigromaculata* (Hai Phong, Vietnam); I – *M. steinegeri* (Taiwan, China); J – *M. sumatrana* (South Sumatra, Indonesia); K – *Micryletta melanops* **sp. nov.** (Lam Dong, Vietnam). Photos by Nikolay A. Poyarkov, Jian-Huan Yang, ParinyaPawangkhanant, Chung-Wei You, and Eki Aprilia Resdiyanti Devung.