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Morphology, phylogeny, and species delimitation of *Micryletta* (Anura: Microhylidae) reveals a new species from Singapore

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http://zoobank.org/CFFC8915-F3A3-4C04-AE4C-381302C03D72

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Academic editor Raffael Ernst | **Received** 07 April 2022 | **Accepted** 02 June 2022 | **Published** 29 June 2022

Citation: Sankar A, Law IT, Law IS, Shivaram R, Abraham, RK, Chan KO (2022) Morphology, phylogeny, and species delimitation of *Micryletta* (Anura: Microhylidae) reveals a new species from Singapore. Vertebrate Zoology 72 457–467. https://doi.org/10.3897/vz.72.e85020

Abstract

The genus *Micryletta*, also known as paddy frogs, ranges across much of south, east, and southeast Asia. Due to their relatively broad distribution and overall morphological similarities, many species have gone undetected until recently, largely owing to the use of molecular data. Consequently, the species diversity within this genus has quadrupled in just three years from three species prior to 2018, to 12 species in 2021, indicating that the systematics of this genus is still poorly understood. As such, we assembled the most comprehensive molecular phylogeny of *Micryletta* hitherto including novel sequences from a previously unsampled population from Singapore to assess the species diversity within this genus. In particular, we investigate the population from Singapore whose specific identity remains in question due to the lack of voucher specimens and genetic material. Our results show that the Singapore population represents a strongly supported and distinct lineage that is most closely related to *M. inornata* sensu stricto from Sumatra, Indonesia. Morphological and species delimitation analyses corroborate its distinction as a new species, which we describe herein as *M. subaraji* sp. nov. This and recent new taxon discoveries in Singapore demonstrate that the biodiversity of the highly urbanized island-state is still far from being fully realized and underscores the need for continued systematic surveys and protection of remaining habitats.

Keywords

Amphibian, Kranji Marshes, *Micryletta subaraji* sp. nov., Systematics, Taxonomy

Introduction

The amphibian genus *Micryletta* Dubois, 1987 (family: Microhylidae), commonly referred to as paddy frogs, is a relatively small genus comprising twelve species, namely *M. aishani* Das, Garg, Hamidy, Smith and Biju, 2019; *M. dissimulans* Suwannapoom, Nguyen, Pawangkhanant, Gorin, Chomdej, Che and Poyarkov, 2020; *M. erythropoda* (Tarkhnishvili, 1994); *M. hekouensis* Liu, Hou, Mo and Rao, 2021; *M. immaculata* Yang and Poyarkov, 2021;

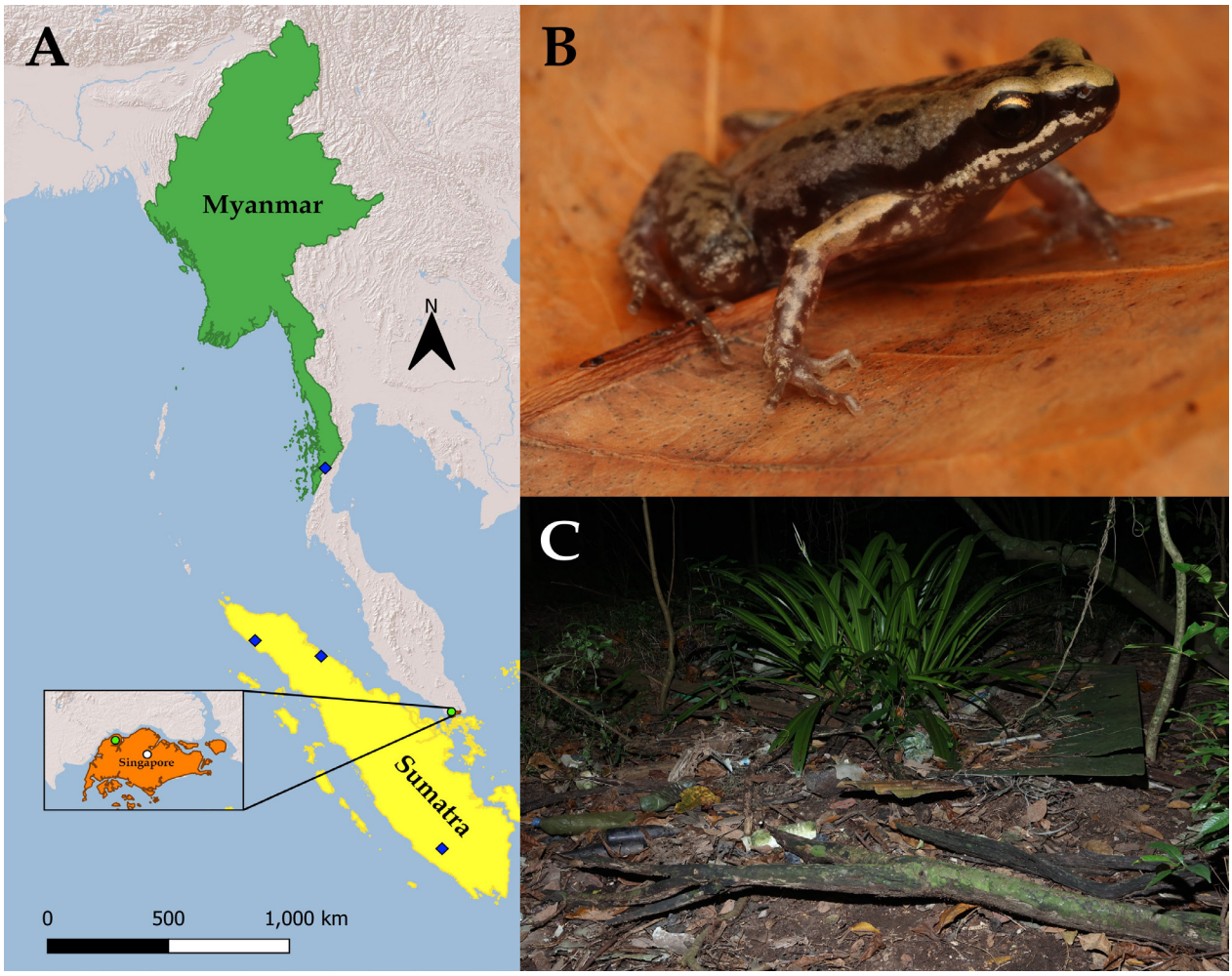

Figure 1. A Distribution of confirmed localities of *Micryletta inornata* s.s. from Sumatra and Myanmar (blue diamonds) and *M.* cf. *inornata* from Kranji Marshes (green circle), and Central Catchment Nature Reserve (white circle) in Singapore. **B** Photo showing *M.* cf. *inornata* in life. **C** Degraded habitat at Kranji Marshes that *M.* cf. *inornata* specimens were detected in. Photos by Law Ingg Thong.

M. inornata (Boulenger, 1890); *M. lineata* (Taylor, 1962); *M. melanops* Poyarkov, Nguyen, Yang, and Gorin, 2021; *M. menglienica* (Yang and Su, 1980); *M. nigromaculata* Poyarkov, Nguyen, Duong, Gorin and Yang, 2018; *M. steinegeri* (Boulenger, 1909); and *M. sumatrana* Munir, Hamidy, Matsui, Kusrini and Nishikawa, 2020. These small-sized terrestrial frogs range from northern Sumatra through the Malay Peninsula, Indochina, southern China (Yunnan and Taiwan), and northeastern India (Frost 2021). Prior to the year 2018, only three species were recognized (*M. erythropoda*, *M. inornata*, and *M. steinegeri*). However, recent studies employing molecular data have led to the discovery of nine additional species between the years 2018–2021 alone (Frost, 2021), thereby significantly improving our understanding of the systematics and evolutionary history of *Micryletta* within a relatively short period of time. These recent discoveries suggest that the species diversity in *Micryletta* is not yet fully realized and underscores the need for additional studies.

Micryletta inornata was described from Sumatra, Indonesia, and is the type species of the genus. Due to morphological similarities with other species of *Micryletta*, *M. inornata* was historically considered a widespread species that occurred in Sumatra and throughout the Malay Peninsula and Indochina. However, a recent study by Alhadi et al. (2019) restricted the distribution of *M. inornata* to the island of Sumatra, resulting in numerous unnamed lineages occurring elsewhere that were eventually described as new species by other authors (Das et al. 2019, Munir et al. 2020, Suwannapoom et al. 2020, Liu et al. 2021, Poyarkov et al. 2021, Yang and Poyarkov 2021). Subsequently, a disjunct distribution of *M. inornata* sensu stricto (s.s.) was discovered in the Tanintharyi Region of southern Myanmar (confirmed using genetic data), where it is sympatric with another species, *M. lineata* (Miller et al. 2021).

In 2019, the occurrence of *Micryletta* was reported for the first time in Singapore (Central Catchment Nature Reserve, CCNR) and was putatively identified as *M.* cf. *inornata* based on cursory morphological examination in the field and live photographs of the specimen taken prior to its release (Law et al. 2019). An additional population was later discovered in the northwestern region of Singapore at Kranji Marshes (Fig. 1A, C). However, due to the absence of voucher specimens and robust comparisons, the specific identity of *Micryletta* in Singapore remains

in question. In this study, we collected for the first time, voucher specimens and genetic material of *M*. cf. *inornata* from Singapore and assembled the most comprehensive molecular phylogeny of *Micryletta* hitherto to assess species diversity and boundaries within this genus, with particular emphasis on the population from Singapore whose specific identity remains uncertain.

Methods

Sampling

We collected 11 specimens during fieldwork in Kranji Marshes, Singapore (1°25.1150'N, 103°43.2641'E; Fig. 1A–C) between January and October 2021 (surveys in the CCNR failed to yield any specimens). The site is dominated by marshy young secondary scrubland (Fig. 1C). Historically, the area was a coconut plantation bounded by mangroves along the Kranji River. With the damming of the river, the surrounding mangroves were converted into freshwater marshes and there is a conservation site approximately 200 m northeast of the site sampled. The site is highly disturbed and is littered with trash and construction rubble (Fig. 1C). Several individuals were found throughout the site calling after rain. We collected and dissected the specimens to extract liver tissue, which was preserved in 100% ethanol. Specimens were fixed in 10% formalin and subsequently preserved in 70% ethanol. All voucher specimens collected in this study are deposited in the Zoological Reference Collection (ZRC) at the Lee Kong Chian Natural History Museum, National University of Singapore. Advertisement calls were recorded using an Olympus LS-100 recorder and a Sennheiser ME-66 microphone.

Morphology and Bioacoustics

We took the following measurements of collected specimens using Mitutoyo digital calipers to the nearest 0.01 mm: SVL (snout-vent length; tip of snout to cloaca), HL (head length; tip of snout to posterior margin of mandible), HW (head width; measured across mandibular articulations), HD (head depth; measured at the occiput), UEW (upper eye-lid width; widest distance measured from upper margin of orbit to medial margin of upper eye lid), EL (eye length; measured across anterior and posterior corners of orbit), IND (internarial distance; distance across medial margins of nostrils), IOD (inter-orbital distance; smallest distance between medial margins of upper eye lid), SL (snout length; tip of snout to anterior-most point of orbit), N-EL (nostril-eye length; posterior margin of nostril to anterior-most pint of orbit), S-NL (snout-nostril length, anterior margin of nostril to tip of snout), FAL (forearm length; base of outer palmar tubercle to posterior margin of elbow inflection), HAL (hand length; base of outer palmar tubercle to tip of third finger), THL (thigh length; cloaca to outer margin of knee inflection), AGL (axilla-groin length; distance between arm and leg insertions), TFL (tibiofibula length; measured across the outer margins of knee and ankle inflections), and FL (foot length; outer margin of ankle inflection to tip of fourth toe).

A Levene's test was performed on each dependant variable (character) to test for equal variance. However, the *p*-values of the Levene's tests were >0.05 for each dependant variable. Therefore, *t*-tests were performed to determine whether the Singapore specimens were statistically different from *Micryletta inornata* s.s. collected from Sumatra following the framework outlined by Chan and Grismer (2021). A principal component analysis was also performed to find the best low-dimensional representation of variation in the data to determine whether morphological variation could form detectable group structure. Measurements for *Micryletta inornata* s.s. were obtained from Alhadi et al. (2019). Measurements (except for SVL) were corrected for body-size variation using an allometric growth model implemented in the R package *GroupStruct* (Chan and Grismer 2022) and all morphological analyses were performed on the size-corrected data. All morphological analyses were performed in R v.3.6.1 (R Core Team, 2014).

Advertisement call recordings were cleaned of background noise using the Noise Reduction tool in Audacity version 2.3.3 (Audacity, GNU General Public License). Noise reduction settings are as follow: Noise Reduction (dB) = 40; Sensitivity=20; Frequency Smoothing (bands) = 3. A total of 12 calls (n=12) from a single individual was analyzed. All calls were recorded at an ambient temperature range of 26–27 ºC. We generated oscillograms and spectrograms using the R package of SEEWAVE (Sueur et al. 2008).

Molecular Analysis

We amplified a ~900 bp fragment of the 16S rRNA mitochondrial gene for four specimens from Singapore (ZRC 1.13323, ZRC 1.13369, ZRC 1.13370, ZRC 1.13389) using the primers 16SD-L (5′-GTRGGCCTAAAAGCAGCCAC-3′), and 16SD- H (5′-CTCCGGTCTGAACTCAGATGACGTAG- 3′) (Evans et al. 2003, Chan et al. 2022). Amplification was done using the following PCR thermal profile: 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 70 s, and a final extension phase at 72 °C for 7 min.

Sequences were assembled and aligned (MUSCLE algorithm) using Geneious v5.6.7 (Kearse et al. 2012). New sequences generated for this study are cataloged under the GenBank accession numbers ON026063–66. Additional published sequences of other *Micryletta* species were obtained from GenBank to supplement the phylogenetic analysis. A maximum likelihood phylogeny was inferred using IQTREE 2 (Minh et al. 2020). The best-fit substitution model was determined using ModelFinder (Kalyaanamoorthy et al. 2017) and branch support was assessed using 1000 ultrafast bootstrap replicates.

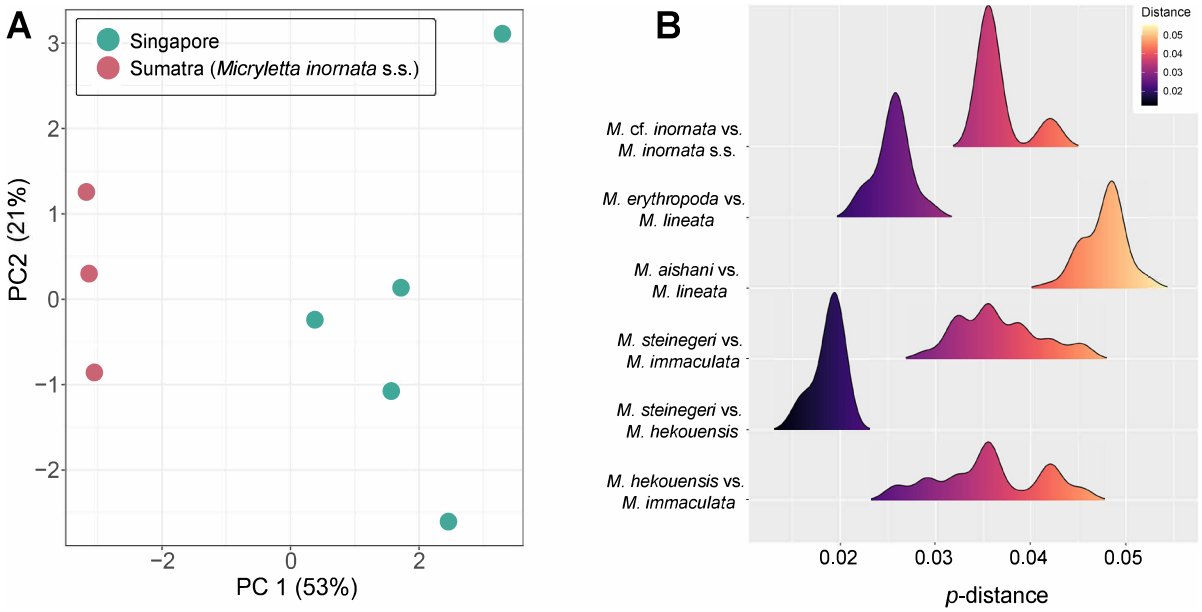

Figure 2. A PCA plot of size-adjusted male specimens of *Micryletta inornata* s.s. from Sumatra (red) and *M.* cf. *inornata* from Singapore (green); **B** density plots comparing uncorrected *p*-distances between closely related *Micryletta* lineages.

We also performed two species delimitation analyses using the programs mPTP v.0.2.4 (Kapli et al. 2017) and ASAP (Puillandre et al. 2021) to determine if the Singapore population could represent a distinct species. For the mPTP analysis, the mitochondrial phylogeny was used as the input tree and the minimum branch length was calculated using the –minbr_auto function. The confidence of delimitation schemes was assessed using two independent MCMC chains at 10,000,000 generations each. The ASAP analysis was performed using the Kimura K80 substitution model (ts/tv=2.0) through the web-server accessible at https://bioinfo.mnhn.fr/abi/public/asap. Uncorrected *p*-distances were calculated in MEGA-X (Kumar et al. 2018) using the Complete Deletion option for missing data.

Results

Morphological Analyses

Raw measurements and summary statistics of all 11 specimens collected in this study are presented in Table 1. The *t*-test revealed that male specimens from Singapore differed significantly ($p < 0.05$) from male specimens of *Micryletta inornata* s.s. from Sumatra in several head-related characters (head width, head length, snout length, nostril-eye length, snout-nostril length, upper eye-lid width) and also tibiofibula length. Of these differences, head width, head length, snout length, snout-nostril length, and upper eye-lid diameter were found to be highly significant ($p < 0.01$; Table 2). Female and juvenile specimens were not assessed due to limited sample size. The PCA analysis showed that the Singapore population is distinctly separated from the *M. inornata* s.s. along PC1, which accounted for 53% of the variation (Fig. 2A).

Phylogenetics, Species Delimitation, and Bioacoustics

Comparisons of uncorrected *p*-distances revealed that *M.* cf. *inornata* from Singapore is 3.5–4.2% divergent from *M. inornata* s.s., which is comparable to divergences between *M. steinegeri* and *M. immaculata* (2.9–4.5%) and between *M. hekouensis* and *M. immaculata* (2.6–4.5%) but higher than divergences between *M. erythropoda* and *M. lineata* (2.3–2.9%) and between *M. steinegeri* and *M. hekouensis* (1.6–1.9%; Fig. 2B). The Singapore population was reciprocally monophyletic with *Micryletta inornata* s.s. from Indonesia with strong support (bootstrap=100; Fig. 3). Similar to Miller et al. (2021), sequences from southern Myanmar were recovered within the *Micryletta inornata* s.s. clade but with weak support. The relationships among other species of *Micryletta* received mixed support and were different in several regards from previous phylogenies (Liu et al. 2021, Poyarkov et al. 2021, Yang and Poyarkov 2021).

The mPTP species delimitation analysis inferred the Singapore population as a distinct species with strong support (average support value = 99; Fig. 4). *Micryletta inornata* s.s., *M. sumatrana*, *M. dissimulans*, *M. nigromaculata*, *M. aishani*, *M. erythropoda*, and *M. lineata* were also corroborated as distinct species with strong support. However, *M. hekouensis*, *M. steinegeri*, and *M. immaculata* were lumped as a single species indicating that the distinction between these species is weak. Similarly, the ASAP analysis also inferred the Singapore population as a distinct species across all partitions. The top-ranked partition further split *M. inornata* s.s. and *M. immaculata*, while lumping *M. steinegeri* and *M. hekouensis* (Fig. 4).

The advertisement call of *Micryletta* cf. *inornata* from Singapore is a pulsatile sweeping metallic call that comprises of an average of 21 pulses (mean=21; range=16–28;

Table 1. Measurements (in mm) of collected *Micryletta* cf. *inornata* from Singapore. Abbreviations are defined in Methods; the asterisk (*) denotes the holotype specimen. Mensural data of juvenile specimens, which are not in the type series, are also provided.

	ZRC 1.13370*	ZRC 1.13369	ZRC 1.13389	ZRC 1.13469	ZRC 1.13470	Mean ± SD (n =5)	ZRC 1.13323	ZRC 1.13466	ZRC 1.13467	ZRC 1.13468	Mean ± SD (n =4)	ZRC 1.13390	ZRC 1.13465	Mean ± SD (n =2)
Sex	Male	Male	Male	Male	Male		Female	Female	Female	Female		Juvenile	Juvenile	
SVL	18.90	17.86	17.73	17.45	18.09	18.01±0.49	23.04	20.69	16.94	18.06	19.68±2.37	15.05	15.40	15.23±0.18
HL	6.23	5.82	5.88	5.97	6.31	6.04±0.19	6.46	6.56	4.99	5.90	5.98±0.62	4.76	5.17	4.97±0.21
HW	6.55	5.92	6.48	6.26	6.17	6.28±0.23	5.91	5.50	4.81	5.25	5.37±0.4	4.86	4.94	4.9±0.04
HD	3.85	3.6	3.35	4.04	3.82	3.73±0.24	2.02	3.32	3.19	2.46	2.75±0.53	2.71	2.50	2.61±0.11
UEW	1.54	1.31	1.43	1.21	1.25	1.35±0.12	1.32	1.14	1.65	1.29	1.35±0.19	1.09	1.20	1.15±0.05
EL	2.39	2.12	2.46	2.12	2.37	2.29±0.14	2.92	2.95	2.45	2.50	2.71±0.23	1.54	2.20	1.87±0.33
IND	1.76	1.45	1.91	1.64	1.57	1.67±0.16	1.41	1.85	1.62	1.84	1.68±0.18	1.27	1.50	1.39±0.12
IOD	2.26	2.31	2.61	2.50	2.00	2.34±0.21	2.96	3.04	2.29	2.70	2.75±0.29	2.67	2.38	2.53±0.15
SL	2.41	2.12	2.26	2.31	2.59	2.34±0.16	2.95	3.30	2.73	2.82	2.95±0.22	2.26	2.88	2.57±0.31
N-EL	1.26	1.26	1.36	1.33	1.40	1.32±0.06	2.38	2.48	2.22	2.34	2.36±0.09	1.96	1.93	1.95±0.02
S-NL	1.15	0.86	0.9	0.98	1.19	1.02±0.13	1.50	1.22	1.14	1.00	1.22±0.18	1.06	1.26	1.16±0.1
FAL	4.23	4.49	4.43	4.27	3.82	4.25±0.23	10.63	8.95	7.23	8.46	8.82±1.22	6.53	7.22	6.88±0.35
HAL	5.13	4.86	4.57	4.37	5.03	4.79±0.28	5.72	5.68	4.16	4.58	5.04±0.68	3.65	3.94	3.8±0.15
THL	8.24	8.24	7.41	7.99	8.04	7.98±0.3	9.12	8.29	7.16	6.89	7.87±0.89	6.81	6.55	6.68±0.13
AGL	6.91	6.53	8.25	9.75	9.08	8.1±1.23	12.5	8.73	8.37	8.27	9.47±1.76	7.4	7.27	7.34±0.07
TFL	8.59	8.15	7.98	7.92	8.01	8.13±0.24	9.47	9.49	7.82	8.11	8.72±0.76	6.78	7.1	6.94±0.16
FL	9.4	8.55	7.37	9.30	10.75	9.07±1.11	7.76	9.96	7.77	8.72	8.55±0.9	7.21	7.38	7.3±0.09

Table 2. Summary statistics of male specimens (mean ± standard deviation) and results of the *t*-test between *Micryletta inornata* s.s. from Sumatra and *Micryletta* cf. *inornata* from Singapore. The *t*-test was performed on the size-adjusted data (see materials and methods for more details). Size-adjustments were applied for all parameters except SVL. Data from Sumatra were obtained from Alhadi et al. (2019). Female and juvenile specimens were not assessed due to limited sample size. Abbreviations are defined in Methods. * = $p < 0.05$; ** = $p < 0.01$

	Mean ± SD (Sumatra, n=3)	Mean ± SD (Singapore, n=5)	*p*-value
SVL	18.1±1.7	18.01±0.49	0.9797
HL	4.83±0.21	6.04±0.19	0.0000**
HW	5.4±0.36	6.28±0.23	0.0050**
UEW	0.97±0.12	1.35±0.12	0.0014**
EL	2.17±0.09	2.29±0.14	0.1962
IND	1.4±0.08	1.67±0.16	0.0603
IOD	2.4±0.24	2.34±0.21	0.6226
SL	1.93±0.12	2.34±0.16	0.0042**
N-EL	1.2±0.22	1.32±0.06	0.0268*
S-NL	0.57±0.09	1.02±0.13	0.0001**
FAL	4.1±0.24	4.25±0.23	0.4344
HAL	4.53±0.37	4.79±0.28	0.0864
TFL	8.67±0.56	8.13±0.24	0.0313*
FL	8.43±0.52	9.07±1.11	0.3709

n=12; Fig. 5). Calls had a mean dominant frequency of 5333.05 Hz (n=12), with a duration of 0.41 s (mean=0.41, range=0.34–0.47, SD=0.04, n=12).

Taxonomy

Results from all morphological, phylogenetic, and species delimitation analyses support the recognition of *Micryletta* cf. *inornata* from Singapore as a distinct species. Applying an integrative taxonomic framework based on multiple lines of support, we describe *Micryletta* cf. *inornata* from Singapore as a new species below:

Micryletta subaraji sp. nov.

https://zoobank.org/581A7F41-1545-4E15-AAC4-EC00-2C048217

Figs 1B, 6, 7

Micryletta inornata Law, Thomas, and Law, 2019: 5

Suggested Common Name. Subaraj's Paddy Frog.

Holotype. ZRC1.13370 (Fig. 6), adult male, collected 8th January 2021, 2130H from Kranji Marshes,

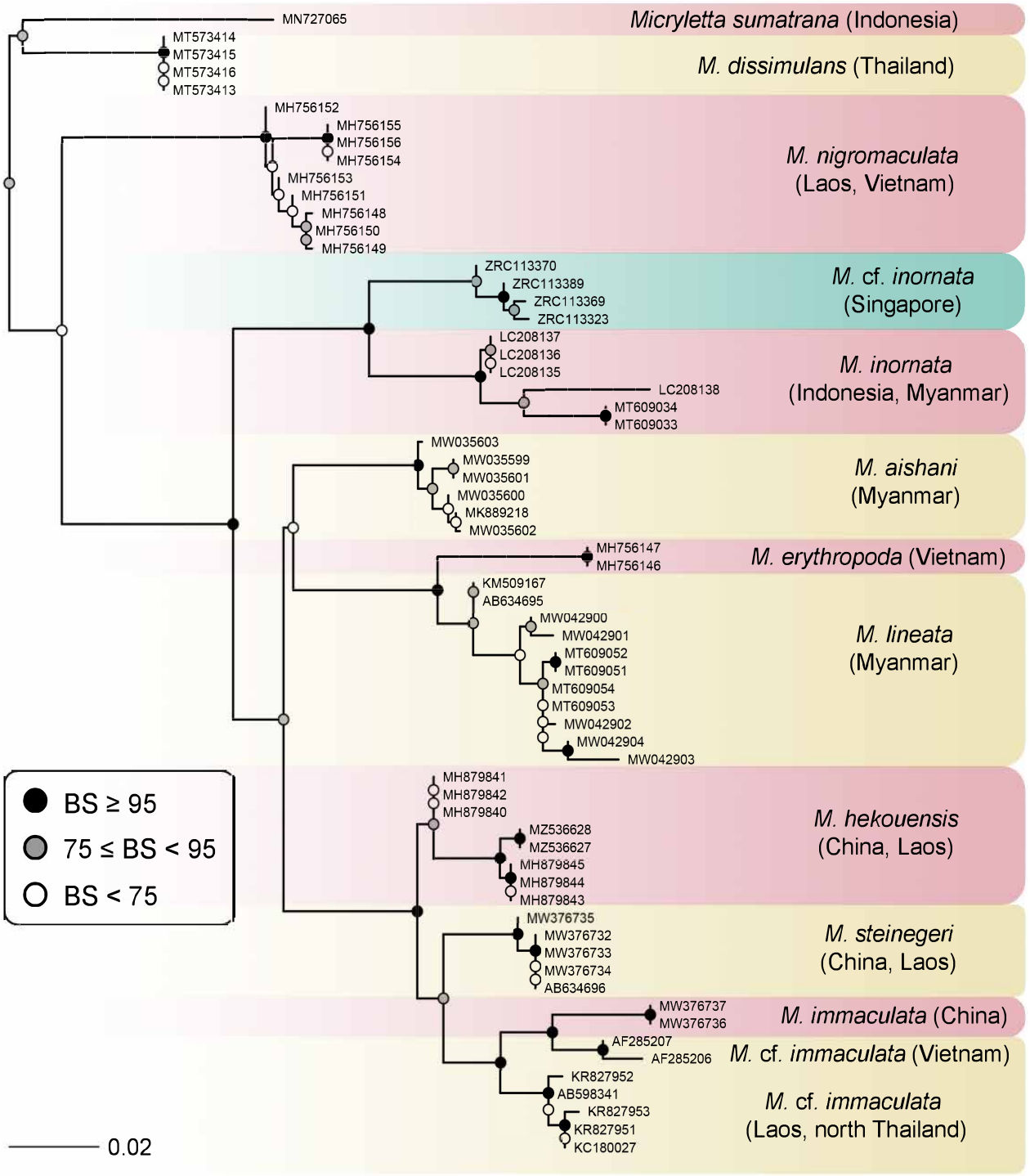

Figure 3. Maximum likelihood phylogeny based on 1,097 bps of the 16S rRNA mitochondrial gene. Nominal species and their regional distribution ranges are labeled to the right of the phylogeny. The clade containing *M.* cf. *inornata* from Singapore is highlighted in green. BS = ultrafast bootstrap support values.

Singapore (1°25.1150'N, 103°43.2641'E, 8 m a.s.l) by Law Ing Sind and Law Ingg Thong.

Paratypes (n=8). ZRC 1.13369, adult male; ZRC 1.13389, adult male; ZRC 1.13469, adult male; ZRC 1.13470, adult male; ZRC 1.13323, adult female; ZRC 1.13466, adult female; ZRC 1.13467, adult female; ZRC 1.13468, adult female; all collected from the same location as the holotype between January and October 2021 by Law Ing Sind, Law Ingg Thong, and Sankar Ananthanarayanan (Fig. 7).

Diagnosis. *Micryletta subaraji* sp. nov. is a member of *Micryletta* based on its sister relationship to *Micryletta inornata* s.s. (Fig. 3). It can be distinguished from other members of the genus by a combination of the following characters: small body size (SVL 18.90 mm in males, 23.04 mm in females), an abruptly rounded snout, lack of webbing between fingers and toes, lack of distinct supratympanic fold, tympanum hidden, the presence of a circular inner metatarsal tubercle, and the absence of an outer metatarsal tubercle. Males of *Micryletta subaraji* sp. nov.

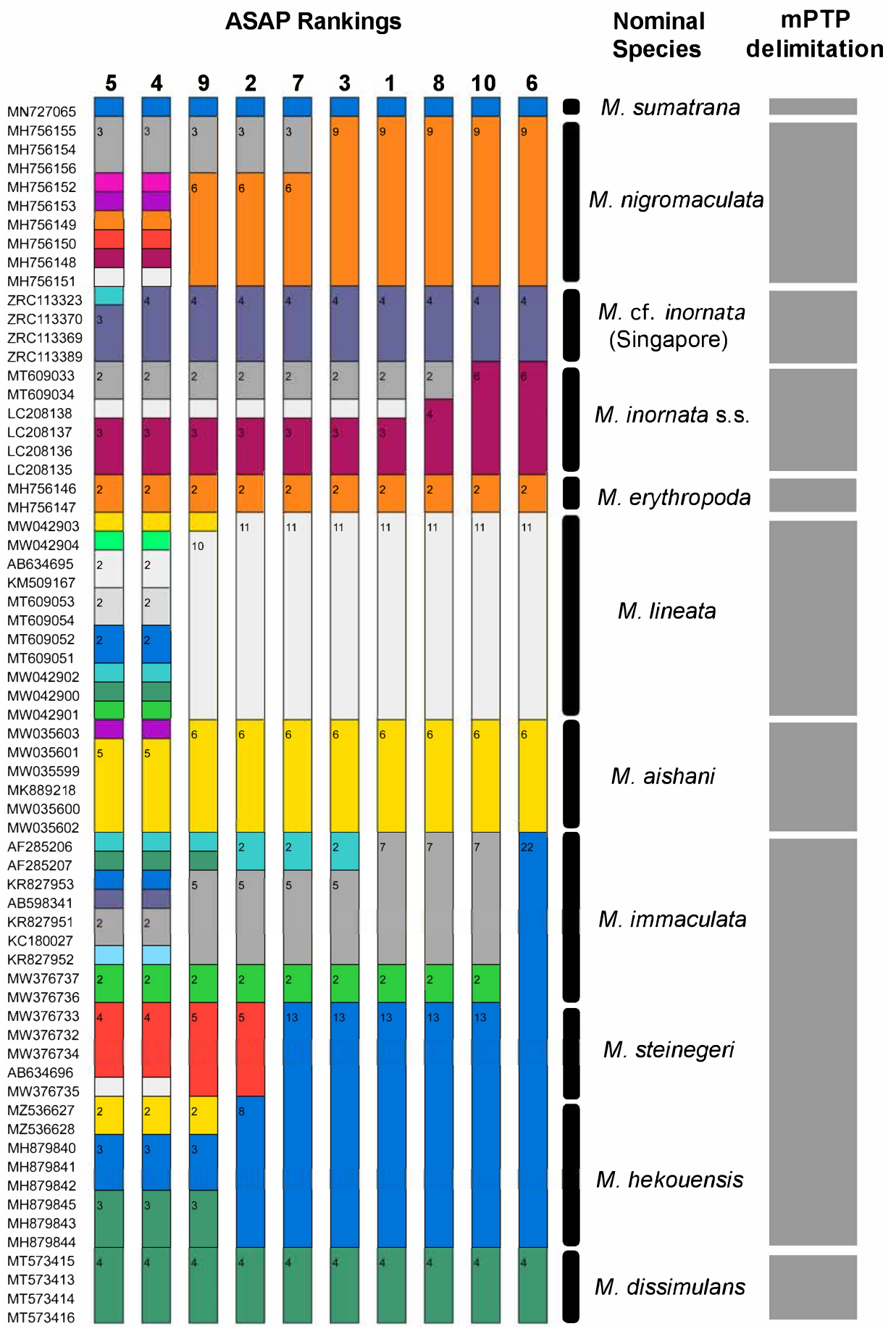

Figure 4. Results of the ASAP and mPTP species delimitation analyses. Rankings for the ASAP analysis are based on ASAP-scores (for more information on ASAP-scores, see https://bioinfo.mnhn.fr/abi/public/asap/FAQ_asap.html). Numbers within bars represent the number of samples contained within each partition.

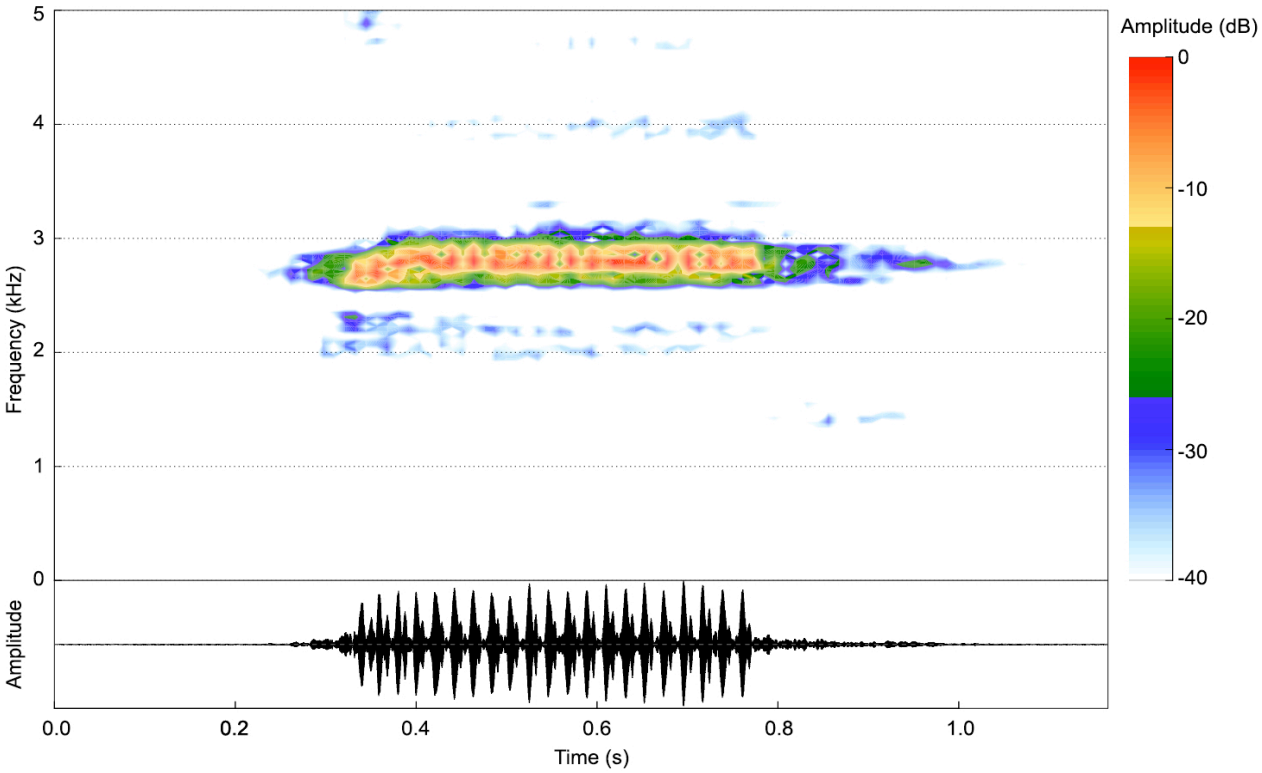

Figure 5. Audio spectrogram (top) and corresponding oscillogram (bottom) for *Micryletta* cf. *inornata* from Singapore.

can be distinguished from males of its sister species *M. inornata* s.s. from their significantly larger head proportions (head width, head length, snout length, nostril-eye length, snout-nostril length, upper eye-lid diameter) and shorter tibiofibula length.

Description of holotype. Adult male, SVL 18.9 mm; habitus slender; head slightly wider than long (HW/HL 1.05), acuminate in dorsal profile with abruptly rounded snout; snout length (SL 2.41 mm) roughly equal to eye diameter (EL 2.39 mm), and slightly longer than interorbital distance (IOD 2.26 mm); nostrils closer to tip of snout (S-NL 1.15 mm) than to anterior tip of eye (N-EL 1.26 mm); interorbital distance larger than internarial distance (IND 1.76 mm); eyes large, 38% of head length; pupil round and moderately dilated; tympanum not visible, and supratympanic fold not present. Skin on dorsal and ventral surfaces smooth; forelimbs and hindlimbs slender; F3>F4>F2>F1, T4>T3>T5>T2>T1; fingertips and toetips lacking in discs; no webbing between fingers and toes; circular basal subarticular tubercles on all fingers and toes; circular supernumerary metacarpal tubercles present at the base of F2, F3, and F4, palmar tubercle well-developed and circular, thenar tubercle and outer metacarpal tubercle circular; inner metatarsal tubercle circular, outer metatarsal tubercle absent (Fig. 6).

Dorsum greyish brown; forelimbs and hindlimbs light brown with dark brown mottling; one dark brown broken vertebral stripe, with broken paravertebral stripes on each side; strong dark brown cephalic mottling in between eyelids A solid black lateral stripe from the tip of snout, past eye, remains unbroken until forearm, becomes a dark mottling between forelimb and hindlimb, where it terminates; a thin cream stripe runs parallel below the eyestripe, starts at nostril, terminates at forelimb. Venter cream with dark brown to grey mottling under sides of belly; inner legs are immaculate and uniformly light cream, tibiofibula is moderately mottled, bottom of tarsus is light brown. Stippling is more apparent under a microscope. Black gular sac (Fig. 6).

Variation. Raw mensural data are presented in Table 1. Males generally have smaller body sizes than females, although there is overlap in the ranges of values (male SVL 17.45–18.90 mm, female SVL 16.94–23.04 mm). Female ZRC1.13323, which is visibly gravid, is the largest specimen in the series. The holotype is largely representative of the type series, and minimal variation is observed in colouration (Fig. 3A, B). Males all possess a black gular sac. Most specimens have visible dark brown mottling on cream venters. However, this mottling is absent or indistinct in juvenile ZRC 1.13390, juvenile ZRC 1.13465, male ZRC 1.13389, and female ZRC 1.13467 (Fig. 7).

Distribution. *Micryletta subaraji* sp. nov. is so far only known from Singapore where it occurs in Kranji Marshes and putatively in the Central Catchment Nature Reserve. It may also occur in other parts of the island where suitable habitat is present.

Etymology. The specific epithet honours the late Mr. Subaraj Rajathurai, who is a pioneer of conservation in Singapore.

Natural History. In addition to the collected specimens, male advertisement calls were heard at an ephemeral pool

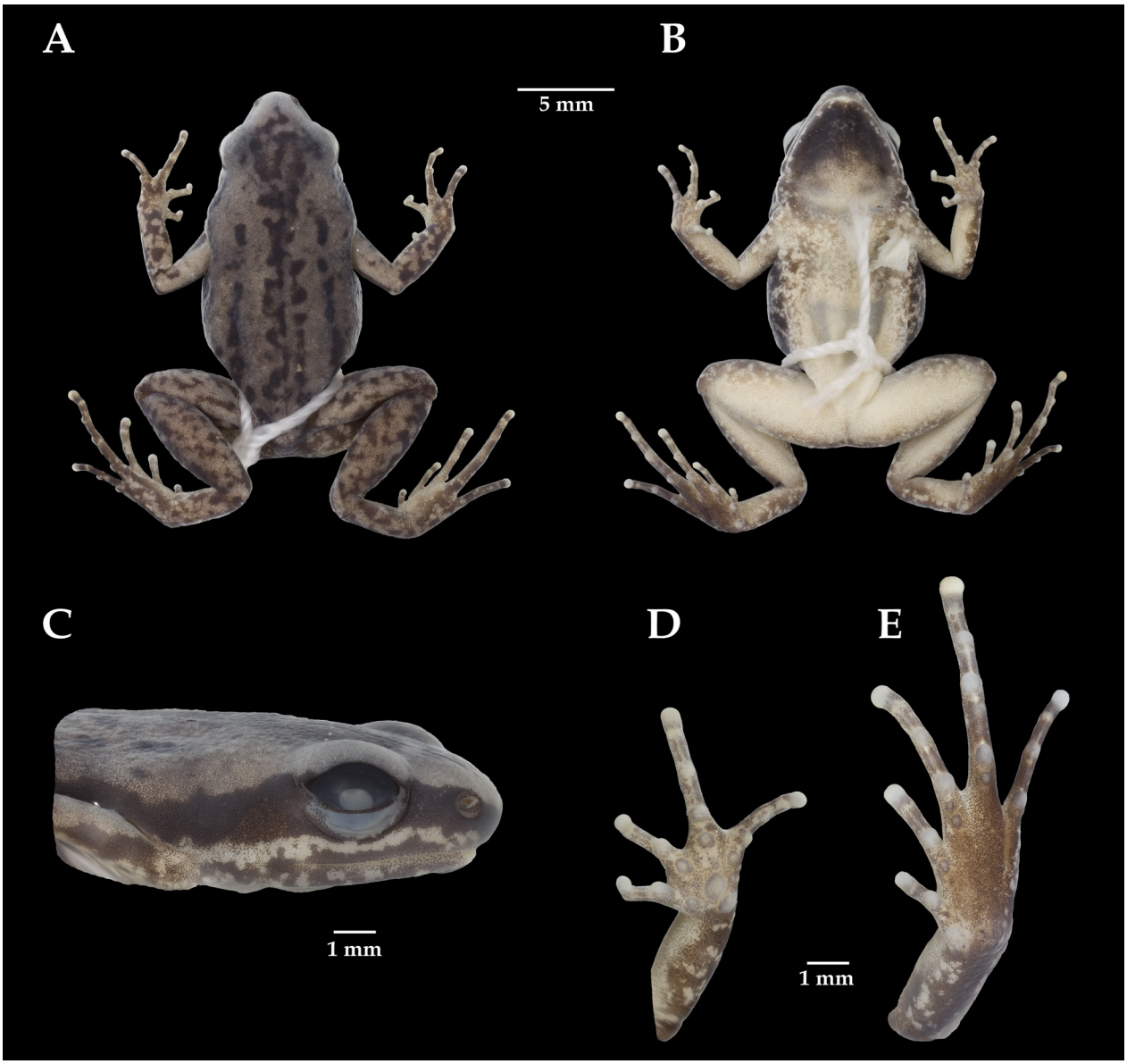

Figure 6. Holotype of *Micryletta subaraji* sp. nov. (ZRC 1.13370, Male) from Kranji Marshes. **A** Dorsal view. **B** Ventral view. **C** Right lateral view of head. **D** Ventral view of left hand. **E** Ventral view of left foot. Photos by Shivaram Rasu

in a depression created by an uprooted tree. The calling individuals were perched amidst dense undergrowth surrounding this puddle. These ephemeral depressions may be used by male frogs as a means to amplify their advertisement calls. Several other Microhylids (both native and introduced) were recorded in sympatry at the type locality including *Microhyla heymonsi*, *Microhyla butleri*, *Microhyla* cf. *mukhlesuri*, and *Kaloula pulchra*.

Discussion

Although our molecular data is based on a single mitochondrial gene, all phylogenetic and species delimitation analyses strongly and unambiguously supported the Singapore population as a new species that is distinct from its sister lineage, *M. inornata* s.s. from Sumatra. This distinction is further corroborated by morphological data. Unfortunately, we were unable to compare advertisement calls because no recordings are available for *M. inornata* s.s. Nevertheless, we present and describe the advertisement call of the new species to facilitate future comparisons. We were also unable to obtain *Micryletta* specimens from the Central Catchment Nature Reserve (CCNR) where it was previously documented but not collected. Based on digital photographs, the CCNR specimen has less prominent dorsal markings and lacks a solid lateral stripe along the side of the head and flank (Law et al. 2019). The CCNR population also appears to be less common than the population at Kranji Marshes. Repeated surveys to CCNR failed to yield a single specimen, whereas frogs were common and abundant at Kranji Marshes. The habitats at these sites are also significantly different—CCNR comprises primary lowland and swamp forests, whereas Kranji Marshes is a highly disturbed, marshy, young secondary scrubland. Based on

Figure 7. Type series of *Micryletta subaraji* sp.nov. from Kranji Marshes in (**A**) dorsal views and (**B**) ventral views. The holotype specimen (ZRC 1.13370) is on the extreme left side of both panels.

differences in color-pattern and habitat preference, it is plausible that the CCNR population is not conspecific with *M. subaraji* sp. nov. However, due to the lack of voucher specimens, we putatively consider the CCNR population as *M. subaraji* sp. nov. until additional data suggest otherwise.

Our phylogeny also indicates that *M. immaculata* is a complex that is not yet adequately understood. This species is currently considered to be endemic to Hainan Island, China (Yang and Poyarkov 2021). However, our results revealed several closely related lineages from Vietnam, Laos, and northern Thailand (Chiang Mai) that we putatively assign to *M.* cf. *immaculata* (Fig. 3). These lineages could either be undescribed species or alternatively, the distribution of *M. immaculata* could be wider than previously thought. Regardless, the *M. immaculata* complex warrants further investigation.

Despite being a small and highly urbanized city-state, the occurrence of *Micryletta* in Singapore was only discovered in 2019 (Law et al. 2019). Numerous significant discoveries across different taxonomic groups have also been made in recent years (Koh and Court 2019, Jusoh et al. 2021, Ye and Wei 2021), suggesting that the biodiversity of Singapore is still far from being fully understood. Taken together, these discoveries highlight the importance of protecting the remaining patches of natural habitat left on the island and underscores the need for continued biodiversity surveys and conservation-related studies.

Acknowledgments

We thank the Singapore National Parks Board for facilitating our research under the permit (NP/RP20-116). We are also grateful to Johnathan Hruska for help with call analyses.

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