

A new limestone-dwelling species of *Micryletta* (Amphibia: Anura: Microhylidae) from northern Vietnam

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Abstract. We report on a new species of the genus *Micryletta* from limestone karst areas in northern Vietnam which is described on the basis of molecular and morphological evidence. *Micryletta nigromaculata* **sp. nov.** is restricted to narrow areas of subtropical forests covering karst massifs in Cat Ba National Park (Hai Phong Province) and Cuc Phuong National Park (Ninh Binh Province) at elevations of 90–150 m a.s.l. In the phylogenetic analyses, the new species is unambiguously positioned as a sister lineage to all remaining species of *Micryletta*. We also discuss genealogical relationships and taxonomic problems within the genus *Micryletta*, provide molecular evidence for the validity of *M. erythropoda* and discuss the taxonomic status of *M. steinegeri*. We suggest the new species should be considered as Data Deficient following the IUCN's Red List categories. A discussion on herpetofaunal diversity and conservation in threatened limestone karst massifs in Southeast Asia is provided.

INTRODUCTION

Paddy frogs of the genus *Micryletta* Dubois, 1987, are a little-known group of microhylids that occurs from southern China, Taiwan, Thailand, Indochina and Myanmar to Nicobar and the

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Comment [1]: The introduction provides a nice overview and is of a good length. Comprehension might be facilitated by adding a figure depicting the different configurations for the genus and subfamily proposed by different authors, but this is just a suggestion.

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Andaman Islands (India), West Malaysia and Sumatra (Indonesia) (Frost, 2018). To date, three species are recognized within the genus: *M. inornata* (Boulenger, 1890) (type locality: Sumatra, Indonesia; distributed in Sumatra, Nicobar and the Andaman Islands, Peninsular Malaysia, Indochina and southern China), *M. steinegeri* (Boulenger, 1909) (distributed in southern Taiwan and China) and *M. erythropoda* (Tarkhnishvili, 1994) (distributed in lowlands of southern Vietnam) (AmphibiaWeb, 2018; Frost, 2018). All these species were initially described within the genus *Microhyla* Tschudi, 1838; however Dubois (1987) erected a new genus *Micryletta*, distinguishing it from *Microhyla* on the basis of a suite of characters including: snout shorter than the eye and eye less prominent (vs. opposite condition in *Microhyla*); distinct tympanum (vs. hidden in *Microhyla*); first finger not reduced (vs. opposite condition in some species of *Microhyla*); digit tips not expanded into disks (vs. expanded in most species of *Microhyla*); and webbing totally absent in *Micryletta* (vs. always present in *Microhyla*) (Dubois, 1987; Bain & Nguyen, 2004).

Owing to morphological conservativeness, biodiversity of the genus *Micryletta* is insufficiently studied and its taxonomy was confusing. For instance, *M. steinegeri*, endemic to Taiwan, was synonymized with *M. inornata* due to the difficulty in distinction between these two species confused with *M. inornata* (Parker, 1928, 1934; Wang et al., 1989). Morphological study by Dubois (1987) supported the validity of *M. steinegeri*, which was followed by Fei et al. (2009, 2010) but rejected by Zhao & Adler (1993), while Matsui & Busack (1985) confirmed synonymy of *Rana gracilipes* Gressitt with *M. steinegeri*. Validity of a subspecies *M. inornata lineata* (Taylor, 1962) described from southern Thailand was not examined by latter studies. Finally, *Microhyla erythropoda* Tarkhnishvili, 1994 described from two specimens from southern Vietnam was assigned to the genus *Micryletta* by Orlov et al. (2002) and Poyarkov et al. (2014), but without providing details on taxonomy of this group.

Works on molecular phylogenetic relationships of the genus *Micryletta* are scarce. Van der Meijden et al. (2007) as well as Pyron & Wiens (2011) confirmed the validity of the genus *Micryletta* and suggested that *Micryletta* is a sister taxon to the group composed of *Microhyla*, *Glyphoglossus* and *Caluella*, though with low values of node support. Matsui et al. (2011) provided an extensive phylogeny of Asian Microhylinae on the basis of 12S rRNA and 16S rRNA mtDNA data, in their tree phylogenetic position of *Micryletta* within Microhylidae is not supported, though the data suggest parphyly of *M. inornata* with respect to *M. steinegeri*. The

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recent phylogenomic work by *Peloso et al. (2016)* also unambiguously places the genus *Micryletta* as a sister taxon to the group composed of *Microhyla*, *Glyphoglossus* and *Caluella*, while a more recent large-scale multilocus phylogeny by *Tu et al. (2018)* on the contrary places *Micryletta* as a sister lineage of the clade joining *Uperodon*, *Phrynella*, *Metaphrynella* and *Kaloula*. Thus, phylogenetic placement of *Micryletta* within Microhyinae is still **contradictory**, and species-level phylogeny of the genus is **still absent**.

During our recent fieldwork in northern Vietnam, in the limestone forests of Hai Phong and Ninh Binh provinces we encountered unusual microhylid specimens which were tentatively identified as *Micryletta* sp. Consequent phylogenetic analysis of the 16S rRNA mtDNA gene revealed that these populations form a lineage sister to all other recognized species of the genus *Micryletta*. Closer morphological examination showed that the specimens from Hai Phong and Ninh Binh provinces are clearly distinguished from other known members of *Micryletta* by a combination of diagnostic morphological features. In the present paper we provide an updated mtDNA-based genealogy of the genus *Micryletta* and describe a new species from northern Vietnam.

MATERIALS AND METHODS

Nomenclatural acts. The electronic version of this article in Portable Document Format (PDF) will represent a published work according to the International Commission on Zoological Nomenclature (ICZN), and hence the new names contained in the electronic version are effectively published under that Code from the electronic edition alone (see Articles 8.5–8.6 of the Code). This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information can be viewed through any standard web browser by appending the LSID to the prefix <http://zoobank.org/>. The LSID for this publication is as follows: urn:lsid:zoobank.org:pub:32150A60-5D04-4116-9816-0ED3E457504A. The online version of this work is archived and available from the following digital repositories: PeerJ, PubMed Central and CLOCKSS.

Sample collection. Fieldwork was conducted from 10 to 22 October 2013 by Nikolay A. Poyarkov in Cat Ba National Park (hereafter – N. P.), Hai Phong Province; and from 8 to 17 June 2017 by Tan Van Nguyen and Tan Nhat La in Cuc Phuong N. P., Ninh Binh Province of northern

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Vietnam. Surveyed localities are shown in Fig. 1. Geographic coordinates and elevation were obtained using a Garmin GPSMAP 60CSx (USA) and recorded in WGS84 datum. All specimens were preserved in 75% ethanol, and muscle tissues were preserved in 95% ethanol for genetic analysis; the holotype specimen was initially fixed in 4% formalin for 24 hours and later preserved in 75% ethanol. Specimens and tissues were subsequently deposited in the zoological collections of the Zoological Museum of Lomonosov Moscow State University (ZMMU), Moscow, Russia, the Duy Tan University (DTU), Da Nang Province, Vietnam, and the Museum of Biology, Sun Yat-sen University (SYS), Guangzhou, China. Comparative materials examined are stored in the herpetological collections of ZMMU and in the Zoological Institute of the Russian Academy of Sciences (ZISP) in St. Petersburg, Russia.

Specimens collection protocols and animal use were approved by the animal operations were approved by the Institutional Ethical Committee of Animal Experimentation of Sun Yat-sen University (certificate number 2005DKA21403-JK issued to Ying-Yong Wang and Jian-Huan Yang). Fieldwork, including collection of animals in the field, was authorized by the Department of Forestry, Ministry of Agriculture and Rural Development of Vietnam (permit number 1461/TCLN-BTTN, issued September 23, 2013).

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

We amplified mtDNA fragments covering partial sequences 16S rRNA mtDNA gene to obtain a 947 bp-length continuous fragment of mtDNA. 16S rRNA gene was widely applied in biodiversity surveys in amphibians (*Vences et al., 2005a, 2005b; Vieites et al., 2009*), and has been used in the most of recent phylogenetic studies on Microhylinae (*Matsui et al., 2011; Peloso et al., 2016; Nguyen et al., in press*). We performed DNA amplification in 20 μ l reactions using ca. 50 ng genomic DNA, 10 nmol of each primer, 15 nMol of each dNTP, 50 nMol additional MgCl₂, Taq PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.1 mM MgCl₂ and 0.01% gelatine) and 1 U of Taq DNA polymerase. Primers used in PCR and sequencing include: L-2188

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(AAAGTGGGCCTAAAAGCAGCCA), 16sL1 (CTGACCGTGCAAAGGTAGCGTAATCACT) and 16H-1 (CTCCGGTCTGAACTCAGATCACGTAGG) (Matsui *et al.*, 2006; 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 TouchDown program from 65 to 55°C reducing 1 degree Celsius every cycle, and extension for 1 min at 72°C, and final extension step for 5 min at 72°C.

PCR products were loaded onto 1.5% agarose gels in presence of ethidium bromide and visualized in agarose electrophoresis. When distinct bands were produced, we purified PCR products using 2 µl of a 1:4 dilution of ExoSapIt (Amersham) per 5 µl of PCR product prior to cycle sequencing. A 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 version 3.1 Sequencing Standard (Applied Biosystems) and 4.2 µl of water. The cyclesequencing used 35 cycles of 10 sec at 96°C, 10 s at 50°C and 4 min at 60°C. We purified the cyclesequencing products by ethanol precipitation. We carried out sequence data collection and visualization on an ABI 3730xl Automated Sequencer (Applied Biosystems). The obtained sequences are deposited in GenBank under the accession numbers MH756146–MH756156 (Table 1).

Phylogenetic analyses. To reconstruct the matrilineal genealogy, we used all 16S rRNA sequences for *Micryletta* available in GenBank and our newly obtained sequences (see Table 1). For outgroups we added sequences of representatives of all currently recognized Microhyalinae genera. In total, we obtained data for 16S rRNA for 36 specimens, which included six sequences of *Micryletta* sp. from Cat Ba Island, three sequences of *Micryletta* sp. from Cuc Phuong N. P., 11 sequences of all other species of *Micryletta* from Thailand, Laos, Vietnam and Taiwan, including topotype specimens of *M. erythropoda* and *M. steinegeri*, 15 outgroup sequences of other Microhyalinae representatives, and a sequence of *Kalophrynus interlineatus* (Blyth) (Kalophryninae) which was used to root the tree (data summarized in Table 1).

We initially aligned nucleotide sequences using ClustalX 1.81 (Thompson *et al.*, 1997) with default parameters, and then optimized them manually in BioEdit 7.0.5.2 (Hall, 1999) and MEGA 6.0 (Tamura *et al.*, 2013). We used MODELTEST v.3.06 (Posada & Crandall, 1998) to estimate the optimal evolutionary models to be used for the data set analysis. The best-fitting model for the 16S rRNA gene fragment was the GTR+G model of DNA evolution as suggested

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by the Akaike Information Criterion (AIC). We determined mean uncorrected genetic distances (p -distances) between sequences with MEGA 6.0.

We inferred the matrilineal genealogy using Bayesian inference (BI) and Maximum Likelihood (ML) approaches. 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 and sampled 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 the frequency of nodal resolution (posterior probability; BI PP) (Huelsenbeck & Ronquist, 2001). We conducted ML analyses using the RAxML web server (<http://embnet.vital-it.ch/raxml-bb/>, Stamatakis et al., 2008); it was used to search ML trees using the gamma model of rate heterogeneity option. We assessed nodal confidence by non-parametric bootstrapping (ML BS) with 1000 pseudoreplicates (Felsenstein, 1985).

In both datasets, we regarded tree nodes with ML BS values 75% or greater and BI PP values over 0.95 to be sufficiently resolved *a priori*. ML BS values between 75% and 50% and BI PP values between 0.95 and 0.90 were regarded as tendencies. Lower values were considered to indicate unresolved nodes (Huelsenbeck & Hillis, 1993).

Morphological description. Specimens of *Micryletta* sp. were photographed in life and after preservation; specimens were euthanized by 20% solution of benzocaine. Measurements were taken using a digital caliper under a light dissecting microscope to the nearest 0.01 mm, subsequently rounded to 0.1 mm. The morphometrics of adults and character terminology follow Poyarkov et al. (2014): (1) snout–vent length (SVL; measured from the tip of the snout to cloaca); (2) head length (HL; measured from the tip of snout to hind border of jaw angle); (3) snout length (SL; measured from the anterior corner of eye to the tip of snout); (4) eye length (EL; measured as the distance between anterior and posterior corners of the eye); (5) nostril–eye length (N–EL; measured as the distance between the anterior corner of the eye and the nostril center); (6) head width (HW; measured as the maximum width of head on the level of mouth angles in ventral view); (7) internarial distance (IND; measured as the distance between the central points of nostrils); (8) interorbital distance (IOD; measured as the shortest distance between the medial edges of eyeballs in dorsal view); (9) upper eyelid width (UEW; measured as the maximum distance between the medial edge of eyeball and the lateral edge of upper eyelid);

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(10) Tympanum length, measured as the maximum tympanum diameter (TMP); (11) forelimb length (FLL; measured as the length of straightened forelimb to the tip of third finger); (12) lower arm and hand length (LAL; measured as the distance between elbow and the tip of third finger); (13) hand length (HAL; measured as the distance between the proximal end of outer palmar (metacarpal) tubercle and the tip of third finger); (14) first finger length (1FL, measured as the distance between the tip and the distal end of inner palmar tubercle); (15) inner palmar tubercle length (IPTL; measured as the maximum distance between proximal and distal ends of inner palmar tubercle); (16) outer palmar tubercle length (OPTL; measured as the maximum diameter of outer palmar tubercle); (17) third finger disk diameter (3FDD); (18) hindlimb length (HLL; measured as the length of straightened hindlimb from groin to the tip of fourth toe); (19) tibia length (TL; measured as the distance between the knee and tibiotarsal articulation); (20) foot length (FL; measured as the distance between the distal end of tibia and the tip of fourth toe); (21) inner metatarsal tubercle length (IMTL; measured as the maximum length of inner metatarsal tubercle); (22) first toe length (1TOEL), measured as the distance between the distal end of inner metatarsal tubercle and the tip of first toe; (23) fourth toe disk diameter (4TDD). Additionally for holotype description we took the following measurements: (24–26) second to fourth finger lengths (2–3FL-O, 4FL-I; for outer side (O) of the second and third, inner side (I) of the fourth, measured as the distance between the tip and the junction of the neighboring finger); (27–30) second to fifth toe lengths (measured as the outer lengths for toes II–IV, as the inner length for toe V; 2–5TOEL). Terminology for describing eye coloration in living individuals is in accordance with *Glaw & Vences (1997)*; subarticular tubercle formulas follow those of *Savage (1975)*. All measurements were taken on the right side of the examined specimen. Sex was determined by gonadal inspection following dissection.

We compared morphological characters of the new species with other members of the genus and comparative data obtained from the literature: *Micryletta inornata* (Boulenger) (*Boulenger, 1890; Taylor, 1962; Bain & Nguyen, 2004*); *M. steinegeri* Boulenger (*Boulenger, 1909; Wang et al., 1989; Fei et al., 2009, 2010*), and *M. erythropoda* (Tarkhnishvili) (*Tarkhnishvili, 1994*).

RESULTS

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Comment [9]: So if it had been vertically oblong this would be the height, and if it had been horizontally oblong, this would be the breadth. I recommend changing to 'horizontal tympanum diameter' so that the measure always retains comparability.

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Comment [10]: In future I strongly recommend against this style of straightened limb measurement, as it (1) damages museum material, (2) can injure live frogs if used in the field, and (3) you can almost never achieve perfect stretch of a limb, meaning that the variance even within a single specimen can be several mm, and between individuals can be considerable and unpredictable.

Phylogenetic relationships. In the final alignment of 16S rRNA gene, of 947 sites 554 were conserved and 346 sites were variable, of which 250 were found to be parsimony-informative. The transition–transversion bias (R) was estimated as 2.62. Nucleotide frequencies were A=33.81%, T=23.23%, C=23.88%, and G=19.04% (data for ingroup only).

The studied 16S rRNA fragment was unable to resolve the genealogical relationships within Microhylinae (see Fig. 2). According to the results of phylogenetic analyses, the newly discovered populations of *Micryletta* sp. from northern Vietnam form a well-supported clade (1.0/100, hereafter node support values are given for BI PP/ML BS, respectively) markedly distinct from all other examined Microhylinae representatives. The *Micryletta* sp. clade is reconstructed as a sister lineage to all other *Micryletta* specimens (1.0/97), the latter also forming a clade (1.0/95) (Fig. 2). Genealogical relationships within this group suggest that specimens from the type locality of *M. erythropoda* (Ma Da Nature Reserve, Dong Nai Province, Vietnam) are clustered with a sample of *Micryletta* sp. from Ranong Province, Thailand (1.0/100) (the latter sample was previously assigned to *M. inornata lineata* in Matsui et al., 2011). This clade is a sister lineage to the group joining all remaining specimens of *Micryletta inornata* and *M. steinegeri*. Evolutionary relationships within the latter group are essentially unresolved with the Taiwanese population of *M. steinegeri* being nested within the radiation of mainland populations of *M. inornata* (see Fig. 2).

Sequence variation. The uncorrected *p*-distances for the 16S rRNA gene fragment are shown in the Table 2. The interspecific distances within *Micryletta* varied from *p*=2.8% (between *M. steinegeri* and *M. inornata*) to *p*=7.7% (between *M. erythropoda* and *Micryletta* sp. from northern Vietnam). Intraspecific distances ranged from *p*=0.7% in *Micryletta* sp. from northern Vietnam (divergence between Cat Ba and Cuc Phuong populations) to *p*=2.2% in *M. inornata* (Table 2). The newly discovered population of *Micryletta* sp. was clearly divergent from all other known species of *Micryletta* and other examined microhylids.

TAXONOMIC ACCOUNT

The newly-discovered populations of microhylids from Cat Ba and Cuc Phuong are clustered with the genus *Micryletta*, forming a divergent lineage sister to all other representatives of the genus examined. Due to both morphological (see below) and molecular differences of the

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Comment [11]: Always love your trees! ☺

My only comment is that the genus *Calluella* is now considered a synonym of *Glyphoglossus*, because that species is deeply nested in all phylogenomic analyses, and it does not make sense to break it into multiple genera; see Tu et al. 2018 (this needs to be examined in more detail in the future). So please change *Calluella* to *Glyphoglossus* in the tree and throughout the text.

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Comment [12]: But this is presumably for a paraphyletic group with respect to *steinegeri*; it might be helpful to indicate the distance also excluding KUHE 35133.

One option would be to designate KUHE 35133 as an unconfirmed candidate species sensu Vieties et al. 2009, which would render *M. inornata* and *M. steinegeri* monophyletic, unless that specimen has already been examined and confirmed to indeed be morphologically assignable to *M. inornata*. The genetic distances here are, of course, quite small, so this might be considered oversplitting, but given the arguments by other authors that *M. steinegeri* is morphologically distinct from *M. inornata*, it seems like it might be justified. I think a discussion of this point is at least warranted.

newly-collected specimens to all currently recognized species in the genus, herein we describe it as a new species of *Micryletta*.

***Micryletta nigromaculata* sp. nov.**

(Figs. 3–6; Table 3)

Holotype. ZMMU A5934, adult male (field ID NAP-06531), collected by Nikolay A. Poyarkov on 15 October 2013 from the limestone evergreen forest (20.8623° N, 106.9988° E, at an elevation of 90 m a.s.l.), Cat Ba National Park, Hai Phong Province, northern Vietnam.

Paratypes. ZMMU A5935–A5948 (14 adult males, field IDs NAP-03343–03348; NAP-03576–03579; NAP-03589–03590; NAP-08445–08446), with collection information same as for the holotype; SYS a007400 (field ID NAP-08444), adult male with collection information same as for the holotype.

Referred materials. DTU 301–302 (two males; field IDs CP.2018.18, CP.2018.31), DTU 303–305 (three gravid females, field IDs CP.2018.19, CP.2018.20, CP.2018.52) collected by Tan Van Nguyen and Tan Nhat La on 3 June 2018 in the secondary forest on limestone (20.2594° N, 105.6928° E, at elevation of 150 m a.s.l.), Cuc Phuong National Park, Ninh Binh Province, northern Vietnam.

Diagnosis. The new species is assigned to the genus *Micryletta* by the following combination of morphological features: small body size; vomerine teeth absent; tympanum small, rounded, externally visible; very prominent subarticular tubercles on fingers and toes; three well-developed metacarpal tubercles; distinct supernumerary palmar and metatarsal tubercles posterior to base of digits; first finger not reduced; digit tips expanded to very small disks and webbing on fingers and toes totally absent (Dubois, 1987; Fei et al., 2009). *Micryletta nigromaculata* sp. nov. is distinguished from all of its congeners by a combination of the following morphological characters: body size small (SVL 18.5–23.0 mm in males, 24.2–25.9 mm in females); body habitus moderately slender; head wider than long; snout obtusely rounded in profile; eye length equal to or shorter than snout length; interorbital distance two times wider than upper eyelid width; tibiotarsal articulation of adpressed limb reaching the level of eye center; dorsal surface slightly granular with small round flattened tubercles; supratympanic fold present, thick, glandular; outer metatarsal tubercle absent; dorsum coloration brown to reddish-brown; dorsum

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with dark-brown irregular hourglass-shaped pattern edged with orange; body flanks brown with dark patches or spots edged with white, a large black blotch in inguinal area on each side; lateral sides of head immaculate reddish brown lacking white patches; venter whitish with indistinct grey pattern; and throat in males whitish with light-grey marbling.

Description of holotype: Adult male, small-sized specimen in a good state of preservation; body habitus moderately slender, body elongated oval-shaped (Figs. 3, 4); head wider than long (HL/HW ratio 84.4%); snout short (SL/SVL ratio 12.1%), rounded in dorsal view (Fig. 3A) and bluntly rounded in profile, slightly projecting beyond lower jaw (Fig. 3C); eyes comparatively large (EL/SVL ratio 12.6%), slightly protuberant in dorsal and lateral views, slightly longer than snout (EL/SL 104.0%) and shorter than the interorbital distance (EL/IOD 86.4%). Top of head flat; canthus rostralis distinct, rounded; loreal region almost vertical, noticeably concave; nostril round, lateral, located closer to the tip of snout than to eye (N-EL/SVL ratio 8.5%) (Fig. 3C); interorbital distance wider than internarial distance (IND/IOD ratio 66.7%), about two times wider than upper eyelid (UEW/IOD ratio 54.5%). Pineal spot absent; tympanum small (TYD/SVL ratio 5.1%), round, relatively indistinct with tympanic rim not elevated above the tympanal area; supratympanic fold thick, rounded, glandular, gently curving from posterior corner of eye towards axilla. Choanae elongated and oval-shaped, widely spaced; upper jaw edentate; vomerine teeth absent; tongue without papillae, roundly spatulate, lacking posterior notch and free behind for 3/4 of its length.

Forelimbs short and slender (FLL/SVL ratio 72.0%); lower arm comparatively long and slender (LAL/SVL ratio 54.1%), hand less than half the length of the forelimb (HAL/FLL ratio 42.7%). Fingers slender (Fig. 3D; Fig. 5A), completely free of webbing, slightly dorso-ventrally flattened, lacking lateral skin fringes; the first finger well-developed, slightly shorter than the second finger (1FL/2FL ratio 74.8%); relative finger lengths: I<IV<II<III; tips of all finger rounded, not expanded to disks; subarticular tubercles on fingers rounded and very prominent, subarticular tubercle formula: 1, 1, 2, 2; nuptial pad absent; three palmar (metacarpal) tubercles: inner metacarpal tubercle distinct, rounded and flat (IPTL/SVL ratio 2.4%); outer metacarpal tubercle elongated, reniform, located on the outer proximal edge of the palm (OPTL/SVL ratio 3.3%); medial metacarpal tubercle large, rounded and prominent, twice the diameter of the inner metacarpal tubercle, located closer to the outer metacarpal tubercle; three rounded and prominent supernumerary palmar tubercles each at the base of fingers II–IV about the same size as inner

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Comment [15]: Give a colour; dark is not informative enough

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Comment [16]: Figure 3 is nice, but the contrast is quite low because the photos are slightly overexposed. This should be easy to fix in photoshop, and would increase the usability of the figure.

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Comment [17]: More informative would be N-EL/N-Snout, if the latter could be measured.

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metacarpal tubercle, a small rounded supernumerary palmar tubercle between medial metacarpal tubercle and the tubercle at the base of finger III, much smaller than metacarpal tubercles.

Hindlimbs slender and comparatively long (HLL/SVL ratio 152.9%), more than two times the length of the forelimb (FLL/HLL 47.1%); tibia long and slender (TL/SVL 51.4%), around one-third of hindlimb length (TL/HLL 33.6%); heels meet when hindlimbs located at right angles to the body, tibiotarsal articulation of adpressed limb reaching the level of eye center; foot slightly longer than tibia length (FL/TL 105.3%). Relative toe lengths: I<V<II<III<IV; tarsus smooth, inner tarsal fold absent; tips of all toes rounded, weakly dilated into small disks, slightly wider than those of fingers (3FDD/4TDD ratio 76.1%); toes completely free of webbing (Fig. 3E; Fig. 5B); subarticular tubercles on toes round and prominent, subarticular tubercle formula: 1, 1, 2, 3, 2; metatarsal tubercle single: inner metatarsal tubercle oval-shaped, prominent, much shorter than the half of first toe (IMTL/1TOEL ratio 36.8%); outer metatarsal and supernumerary metatarsal tubercles absent.

Skin texture and skin glands: Dorsal surface of head and body slightly granular with few small round low tubercles and granules evenly scattered being more prominent in the posterior part of dorsum, dorsal surfaces of forelimbs smooth, dorsal surfaces of hindlimbs covered by irregularly scattered flat tubercles and pustules; flanks of body and lateral sides of head smooth, with small granules present only in axillary region; upper eyelid without superciliary spines; supratympanic fold thick and glandular; ventral side of body and limbs smooth. Cloacal opening unmodified, directed posteriorly, at upper level of thighs.

Coloration in life: Dorsum coloration in life reddish-brown (Fig. 3A, Fig. 4); dorsal surfaces of forelimbs light brownish-orange on upper arms, reddish-brown on lower arms, dorsal surfaces of hindlimbs slightly darker and tan-brownish in coloration; dorsal surfaces with distinct dark pattern: forehead and snout lighter; an distinct light-brownish interorbital bar runs transversally across the head between the medial parts of upper eyelids; interorbital bar forms a very distinct broad V-shaped figure across the head running posteriorly forming irregular hourglass-shaped dark-brown pattern; two smaller blotches in scapular region; dark pattern on dorsum edged with thin light-brown to orange line; head laterally dark red-brown, supratympanic fold black ventrally, edged with light cream-beige thin line dorsally which continues to upper eyelid and canthus rostralis (Fig. 4); flanks with white speckling and characteristic large black patches edged with thin white lines; larger black blotches located at axillary and groin areas, the

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Comment [19]: is there precedence for using 'feebly' in this context? It reads weird when there are good alternatives that also mean 'slightly' or 'weakly'.

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Comment [20]: Is this really alive, or freshly dead? Unimportant, really, but it looks dead.

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Comment [21]: I would call this mottled caramel and mocha; you can be more precise with your colouration terminology.

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Comment [22]: This is remarkably similar to the pattern described in Rakotoarison et al. 2017 for numerous *Stumpffia* species; maybe look at the discussion of that paper where colour patterns are remarked upon.

latter reaching the sacral area; fingers and toes dorsally beige with indistinct brownish mottling, venter whitish, with indistinct light grey marbled pattern on throat and chest (Fig. 3B); iris dark brown with golden speckles in the upper and lower thirds.

Coloration in preservative: After preservation in formalin and storage in ethanol, the general coloration pattern did not fade, dorsal coloration changed to darker greyish-brown, ventral surface of chest, belly, limbs turned whitish-beige; dorsal pattern, dark spots on flanks not changed, dark brown pattern changed to lighter brown; iris coloration faded and turned completely dark.

Measurements of holotype (all in mm): SVL 22.7; HL 6.9; SL 2.7; EL 2.9; N-EL 1.9; HW 8.1; IND 2.2; IOD 3.3; UEW 1.8; TYD 1.2; FLL 16.3; LAL 12.3; HAL 7.0; 1FL 2.0; 2FL 2.7; 3FL 4.3; 4FL 2.4; IPTL 0.6; OPTL 0.8; 3FDD 0.5; HLL 34.7; TL 11.7; FL 12.3; IMTL 0.7; 1TOEL 2.0; 2TOEL 3.3; 3TOEL 5.0; 4TOEL 6.9; 5TOEL 3.1; 4TDD 0.7.

Variation and sexual dimorphism. Individuals of the type series and the referred materials are generally quite similar in appearance and agree well with description of holotype, but show certain variation in coloration (Fig. 6). Dorsal color may vary from bright reddish-brown (Fig. 6A, B) to ochre-brown and light brown (Fig. 6C) and purplish brown (Fig. 6D). Dark dorsal pattern as well as size and position of black blotches on flanks varies a lot, dark spots in sacral area may be connected (Fig. 4; Fig. 6C) or disconnected (Fig. 6A, B) from the dark spot at groin, or may be absent in females (Fig. 6D). Variation in size and body proportions of the type series and referred materials is given in Table 3. Females are larger than males: SVL 18.5–23.0 mm in males ($N = 22$) and 24.2–25.9 mm in females ($N = 3$). Females have comparatively larger body swollen with eggs, and comparatively shorter forelimbs (FLL/SVL mean ratio 77.4% (66.7%–84.1%, $N = 22$) in males vs. 66.4% (62.4%–68.9%, $N = 3$) in females). Males with single internal vocal sac. Skin texture appears to be less tuberculate in preservative than in life.

Distribution and biogeography: The presently known distribution of *Micryletta nigromaculata* **sp. nov.** is shown in Fig. 1. To date, the new species is known from limestone karst areas covered by primary tropical forest in Cat Ba N. P., Hai Phong Province, and by secondary tropical forest in Cuc Phuong N. P., Ninh Binh Province at elevations 90–150 m a.s.l. Northern Vietnam has one of the world largest areas of limestone landscapes, covered by specific limestone vegetation (Fenart *et al.*, 1999; Day & Ulrich, 2000). The currently known range of *Micryletta nigromaculata* **sp. nov.** is divided by the vast lowlands of the Red River valley, an

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Comment [23]: All of this except the extra toe measurements are given in Table 3, so maybe just write 'For comparative measurements, see Table 3. Additionally: XXX' to reduce redundancy.

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Comment [24]: The figure should cite the sources of the basemaps.

important biogeographic border in Indochina (Bain & Hurley, 2011; Yuan et al., 2016); our phylogenetic analysis estimates genetic divergence between the Cat Ba and Cuc Phuong populations at 0.7% (see Table 2). It is anticipated that *Micryletta nigromaculata* sp. nov. also occurs in the adjacent limestone karsts of northern Vietnam; in particular, records from Quang Ninh, Lang Son and Bac Giang provinces of northeastern Vietnam, as well as from Hoa Binh, Ha Nam and Thanh Hoa provinces of northwestern Vietnam are anticipated.

Natural history notes: Our knowledge on the biology of *Micryletta nigromaculata* sp. nov. is scarce; the species appears to be closely associated with karstic habitats. In Cat Ba N. P. (Hai Phong Province) during a two-week survey in October 2011, specimens were only recorded from a small patch of limestone outcrops ca. 20 m in diameter, near a large limestone karst cliff and a small temporary body of water. Frogs were observed from 16:00–20:00 h hiding between small pieces of limestone rocks. Despite intensive search from 10 to 22 of October 2013, no additional specimens of the new species were recorded from other areas in Cat Ba N. P. In Cuc Phuong N. P. (Ninh Binh Province) specimens were found at night between 19:00–23:30 h near cave entrances and in valleys surrounded by limestone cliffs, relatively near to water sources.

Surrounding habitat was limestone karst covered with primary polydominant tropical forest with multi-layered canopy and an abundance of lianas, with occasional trees of *Streblus macrophyllus* (Moraceae), *Terminalia myriocarpa* (Combretaceae), *Parashorea chinensis* (Dipterocarpaceae), and *Tetrameles nudiflora* (Tetramelaceae) (in Cat Ba N.P.) or secondary forest (in Cuc Phuong N.P.). Reproduction biology, including advertisement call, tadpole morphology, as well as diet of the new species remains unknown.

Other species of anurans recorded syntopically with the new species at the type locality included *Polypedates megacephalus* Hallowell, *P. mutus* (Smith), *Theloderma albopunctatum* (Liu & Hu), *Liuixalus calcarius* Milto, Poyarkov, Orlov & Nguyen, *Philautus catbaensis* Milto, Poyarkov, Orlov & Nguyen, *Hyla chinensis* Günther, *Microhyla butleri* Boulenger, *M. heymonsi* Vogt, and *Micryletta* cf. *inornata* (Boulenger). In Cuc Phuong National Park (Ninh Binh Province) the new species was recorded in sympatry with *Occidozyga martensii* (Peters), *Leptobrachium guangxiense* Fei, Mo, Ye & Jiang, *Ophryophryne microstoma* Boulenger, *Glyphoglossus (Calluella) guttulatus* (Blyth), *Microhyla heymonsi* Vogt, *Micryletta* cf. *inornata* (Boulenger); *Rana johnsi* Smith; *Sylvirana* cf. *annamitica* Sheridan & Stuart; *Raorchestes* cf.

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Comment [25]: Calluella as subgenus or as former synonym? If the latter, this should be 'ex-Calluella' or 'formerly Calluella' or '=Calluella'

menglaensis (Kou); *Theلودerma albopunctatum* (Liu & Hu), and *T. annae* Nguyen, Pham, Nguyen, Ngo & Ziegler.

Genetic divergence. The new species is markedly distinct in mtDNA sequences from all congeners for which comparable sequences are available (mitochondrial gene 16S rRNA; uncorrected genetic distance $\geq 5.7\%$) and is reconstructed as a sister lineage with respect to all other examined members of the genus *Micryletta*.

Comparisons. *Micryletta nigromaculata* **sp. nov.** can be distinguished from all other congeners by external morphology and coloration, including presence of characteristic black patches on flanks and the hourglass-shaped irregular dark pattern on dorsum edged with thin orange line. From *Micryletta erythropoda* (Tarkhishvili, 1994) (type locality in Dong Nai Province, known from lowlands of southern Vietnam) the new species can be distinguished by having generally smaller size in males (SVL 18.5–23.3 mm vs. up to 30 mm in *M. erythropoda*); by lacking outer metatarsal tubercle (vs. present in *M. erythropoda*); by having comparatively longer hindlimbs with tibiotarsal articulation of adpressed limb reaching the level of eye center (vs. reaching the level of the posterior edge of tympanum in *M. erythropoda*); by having dorsal surface feebly granular with small round flattened tubercles (vs. rather smooth dorsum in *M. erythropoda*); dorsum coloration brown to reddish-brown (vs. grey or beige to saturated ochre or brick-red in *M. erythropoda*); dorsum pattern with dark-brown irregular hourglass-shaped pattern edged with orange line and with two large black blotches in inguinal area (vs. extremely variable and formed by more or less dark contrasting spots on reddish background in *M. erythropoda*); lateral sides of head reddish brown without white patches (vs. dark brown with white spotting in *M. erythropoda*); flanks brown with dark patches or spots edged with white (vs. dark brown to grey with white patches in *M. erythropoda*); venter whitish with grey pattern (vs. brownish with violet tint in *M. erythropoda*).

Micryletta nigromaculata **sp. nov.** can be distinguished from *M. inornata* (Boulenger, 1890) (type locality in Deli, Sumatra; distributed through Malayan Peninsula to Myanmar, Indochina and southernmost China) by eye length equal or shorter than snout length (vs. snout shorter than the eye in *M. inornata*); interorbital distance two times wider than upper eyelid width (vs. interorbital space just a little broader than the upper eyelid in *M. inornata*); dorsum coloration reddish-brown (vs. dark brown to violet in *M. inornata*); dorsum pattern with dark-brown irregular hourglass-shaped pattern edged with orange line and with two large dark spots in

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Comment [26]: Very nice detail!

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Comment [27]: See above

inguinal area (vs. more or less spotted or marbled with black blotches or longitudinal stripes in *M. inornata*); side of head dark-brown without white patches (vs. black with a series of white spots along the upper lip in *M. inornata*); flanks brown with dark patches or spots edged with white (vs. usually dark brown with white patches in *M. inornata*); venter whitish (vs. lower parts brown in *M. inornata*); throat in males whitish with light-grey marbling (vs. throat of males black in *M. inornata*).

Micryletta nigromaculata **sp. nov.** can be distinguished from *M. steinegeri* (Boulenger, 1909) (endemic to Taiwan) by having comparatively longer limbs with tibiotarsal articulation of adpressed limb reaching the level of eye center (vs. reaching the level of tympanum in *M. steinegeri*); dorsum coloration brownish to reddish-brown (vs. dark grey to violet in *M. steinegeri*); dorsum pattern with dark-brown irregular hourglass-shaped pattern edged with orange line and with two large dark spots in inguinal area (vs. inguinal dark spots absent, dorsum with irregular dark blotches or speckles in *M. steinegeri*); side of head uniform brown without white patches (vs. grey-brown with a series of white spots in *M. steinegeri*); body flanks with dark patches or spots edged with white (vs. flanks usually grey brown with dark marbling in *M. steinegeri*); venter whitish (vs. venter pinkish to orange in *M. steinegeri*).

Etymology: Specific epithet “*nigromaculata*” is an adjective in the nominative case, feminine gender, derived from Latin words “*niger*” for “black” and “*maculatus*” for “spotted”, in reference the characteristic black blotches on flanks in the new species.

Recommended vernacular names: We recommend “Black-spotted Paddy Frog” as the common English name of the new species and the common name in Vietnamese as “*Nhái bầu hông đen*”.

Conservation status: *Micryletta nigromaculata* **sp. nov.** is to date known only from two National Parks in northern Vietnam; in both localities frogs were recorded from very narrow specific limestone-associated habitats. It is important to notice that karst massifs in Vietnam, as well as in other parts of Southeast Asia, are facing ongoing severe threats from intensive deforestation and cement manufacturing; their continued exploitation for limestone cannot be stopped (Clements *et al.*, 2006). This may be the major threat for the new species. However the actual distribution and population status of *Micryletta nigromaculata* **sp. nov.** are unknown and additional surveys in other limestone karst areas of northern Vietnam are essential for elucidating the biology of the new species and clarifying its conservation status. Given the available

information, we suggest *Micryletta nigromaculata* sp. nov. be considered as a Data Deficient (DD) species following IUCN's Red List categories (IUCN, 2016).

DISCUSSION

Our study provides an updated mtDNA genealogy and a new data on diversity of the genus *Micryletta*, which was not studied in detail in recent works on Microhylidae phylogenetics. The key study by Matsui *et al.* (2011) based on 12S–16S rRNA mtDNA fragment failed to recover phylogenetic placement of *Micryletta* within Microhylidae and concluded that this genus should be removed from the subfamily Microhyliinae to form a distinct monotypic subfamily. These conclusions were not supported by consequent studies used multilocus phylogenetic approach, which all strongly suggested placement of *Micryletta* within Microhyliinae radiation as a sister taxon to the group composed of *Microhyla*, *Glyphoglossus* and *Calluella* (Peloso *et al.*, 2016), or as a sister lineage of the clade joining *Uperodon*, *Phrynella*, *Metaphrynella* and *Kaloula* (Tu *et al.*, 2018).

Matsui *et al.* (2011) based on analyses of three specimens of *Micryletta* further showed that *M. inornata* was paraphyletic with respect to *M. steinegeri*, and argued that a sample of *Micryletta* sp. from Ranong Province in southern Thailand (which they identified as *M. i. lineata*) is more divergent than *M. inornata* from northern Thailand and *M. steinegeri* from Taiwan. Our study revealed a previously unknown species of *Micryletta* in northern Vietnam, which is proposed as a sister lineage with respect to all other examined populations (see Fig. 2). We also analyzed genealogical relationships between 11 samples of *Micryletta* from Vietnam, Laos, Thailand and Taiwan, including two topotype specimens of *M. erythropoda* from southern Vietnam. Our data showed that these specimens cluster with *Micryletta* sp. from Ranong Province and together they form a sister lineage with respect to other populations of *M. inornata* from Indochina and Taiwan. This lineage is clearly divergent from other *M. inornata* populations (5.8%–6.2% in 16S rRNA gene; see Table 2) suggesting that *M. erythropoda* represents a distinct species, which occurs in lowlands of southern Vietnam and, possibly, also in southern Thailand. If identification by Matsui *et al.* (2011) is correct, the name *M. inornata lineata* (Taylor, 1962) should have the priority over *M. erythropoda* Tarkhishvili, 1994. However, inclusion of topotype specimens of *M. inornata lineata* from Nakhon Si Thammarat Province of Thailand is required to revise this problem.

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Comment [28]: For me, this species could easily qualify as Endangered. It is known from two locations, the Extent of Occurrence is (currently) considerably under 5000 km², and the habitat is under known threats that are on-going despite protection status. That is B1ab(iii) Endangered.

The IUCN is currently trying to assess as many things as possible, based on whatever limited knowledge is available. In this case, I think it can be argued that there is enough knowledge to at least give an early assessment. Any status is better than DD, because policy-makers treat DD-species as though they don't exist.

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Comment [29]: See above, Calluella = Glyphoglossus.

In our phylogeny (see Fig. 2) *M. steinegeri* from Taiwan was nested within the radiation of *M. inornata* from mainland Indochina; the Taiwanese sample was only slightly divergent from *M. inornata* (2.8%; see Table 2), the latter was comprised of several moderately divergent lineages. The revision of *M. inornata* – *M. steinegeri* group is currently not possible due to the lack of comparative materials from the type locality of *M. inornata* from Sumatra. Due to the wide range of *M. inornata* sensu lato (from northeast India through Myanmar to Indochina, Malay Peninsula and Sumatra) additional materials and further studies on many populations, especially from Sumatra, are critically required to solve taxonomic problems in this group.

Our study provides new evidence for previously unknown diversity of herpetofauna of karstic areas in Northern Vietnam. Previous studies in limestone massifs of Cat Ba Island in Ha Long Bay uncovered two new species of frogs (Milto *et al.*, 2013) and one new species of gecko (Ziegler *et al.*, 2008) all of which are endemic to the island and strongly associated with karst habitats. Cuc Phuong National Park and adjacent limestone massifs are also known for karst-associated endemism with a new species of gecko discovered from karst formations in this area (Ngo & Chan, 2011). Limestone karst massifs in northern Vietnam are divided by the Red River valley, an important biogeographic border in northern Indochina (Bain & Hurley, 2011; Geissler *et al.*, 2015; Yuan *et al.*, 2016). The discovery of *M. nigromaculata* population in Cuc Phuong National Park, on the other side of the Red River valley (see Fig. 1) provide further evidence for interconnection of limestone karst herpetofauna in northern Vietnam. Despite certain divergence in mtDNA 16S rRNA gene (0.7%), overall morphological similarity of the Cuc Phuong and Cat Ba populations of *M. nigromaculata* suggest they belong to a single species.

CONCLUSIONS

Limestone karst areas are recognized as arks of highly endangered though still insufficiently studied biodiversity. Unique geological structure of karst massifs, formed by erosion and subterranean water drainages create numerous humid microrefugia with stable environmental conditions, which serve as an important environmental buffer for small vertebrates during periods of climate change (Clements *et al.*, 2006; Glaw *et al.*, 2006). The complex terrain of isolated karstic hills and caves create multiple ecological niches what along with their highly fragmented habitat-island nature result in high degrees of site-specific endemism within, and diversity among them (Oliver *et al.*, 2017; Grismer *et al.*, 2018). Limestone karsts are also known

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as important "biodiversity arks" for both surface and cave faunas, yet karstic regions are rapidly becoming some of the most imperiled ecosystems on the planet (Clements *et al.*, 2006; Grismer *et al.*, 2016a, 2016b, 2018; Luo *et al.*, 2016; Suwannapoom *et al.*, 2018). South-east Asia harbours more limestone karsts than anywhere else on earth (Day & Ulrich, 2000) with numerous new species including relic lineages of amphibians and reptiles being discovered from limestone areas (e.g. see discussions in Milto *et al.*, 2013; Grismer *et al.*, 2014; Grismer & Grismer, 2017; Grismer *et al.*, 2016a, 2016b, 2017, 2018; Nazarov *et al.*, 2014, 2018; Connette *et al.*, 2017; Suwannapoom *et al.*, 2018 and references therein). Ironically, though acting as major biodiversity hotspots, limestone karsts are critically endangered due to unregulated quarrying mostly for cement manufacturing which is the primary threat to the survival of karst-associated species (Grismer *et al.*, 2018); their continued exploitation for limestone cannot be stopped (Clements *et al.*, 2006). Until karst habitats in Vietnam are thoroughly investigated, a significant portion of this country's herpetological diversity will remain underestimated and unprotected. Our study thus calls for urgent focused survey and conservation efforts on karst herpetofauna in Southeast Asia and in Vietnam in particular.

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