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NEW SYSTEMATIC INSIGHTS INTO *Micryletta erythropoda* (TARKHNISHVILI, 1994) (ANURA: MICROHYLIDAE) WITH A FIRST REPORT FROM LAVA CAVES IN VIETNAM

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Abstract
Field surveys conducted in 2020 and 2023 within the lava caves of Dak Nong UNESCO Global Geopark, Dak Nong Province, Vietnam, resulted in the collection of nine specimens of the genus *Micryletta*. Molecular and morphological analyses confirmed that these specimens belong to *M. erythropoda*. This study represents the second documented occurrence of *M. erythropoda* outside its type locality in Vietnam and the first known instance of the species inhabiting deep lava caves. Additionally, we provide the first detailed description of male *M. erythropoda* specimens, contributing new insights into the species' morphology and habitat preferences.

***Keywords*:** Amphibians, lava cave, morphological analyses, new record, phylogenetics, systematics

Introduction

The genus *Micryletta* Dubois, 1987, commonly referred to as paddy frogs, is a small genus consisting of thirteen recognized species. Its distribution extends from northeastern India, through China, extending southward to Vietnam, Thailand, Malaysia, Singapore, and Sumatra, Indonesia (Frost 2024, Nguyen *et al*. 2024). Tarkhnishvili (1994) originally described *M. erythropoda* (initially named *Microhyla erythropoda*) based on two female specimens collected from Ma Da Forest in Ma Da (Vinh Cuu) Nature Reserve, Dong Nai Province, Vietnam. Subsequent reports have indicated the

species' presence in various provinces of Vietnam, including Kon Tum, Gia Lai, Dak Lak, Dak Nong, Binh Phuoc, Ba Ria-Vung Tau, Tay Ninh, An Giang, and Kien Giang. However, these accounts lack precise locality details, comprehensive morphological descriptions, and genetic verification (Vassilieva *et al*. 2016, Poyarkov *et al*. 2021). Currently, *M. erythropoda*, like most of its congeners, is known to inhabit terrestrial environments, with no previous evidence of cave or subterranean habitation.

During field surveys in lava caves of Dak Nong UNESCO Global Geopark, Dak Nong Province, Vietnam, we collected nine specimens of *Micryletta*. Molecular and morphological analyses confirmed that this lava cave population belongs to *M. erythropoda*. This study represents the second comprehensive record of the species outside its type locality in Vietnam, along with the first description of male specimens. Additionally, it documents the first known occurrence of *M. erythropoda* in lava tube caves.

Material and Method

Lava cave surveys. Field surveys were conducted in 13 lava caves including caves C0, C1, C2, C3, C4, C6.1, C7, C8, P4, P6, P8, P20, and PT06, located in Dak Nong UNESCO Global Geopark, Krong No District, Dak Nong Province, Vietnam (Fig. 1), in 2020 and 2023. Frogs were collected by hand and photographed using Canon M50 and Canon T7 cameras equipped with 60 mm and 100 mm macro lenses. The specimens were fixed in 90% ethanol and subsequently transferred to 70% ethanol for storage (Crottini *et al.* 2014, Liu *et al.* 2021a, Yang & Poyarkov 2021). Liver tissue samples were preserved in absolute alcohol for molecular analysis. Environmental conditions inside the cave were measured using portable devices: light intensity was recorded with a Digital Lux Meter (Benetech, China), while temperature and humidity were monitored with a Kestrel 2000 Weather Meter (KestrelMeters, USA).

Phylogenetic study. Liver tissue samples were utilized for genomic DNA extraction following the CTAB method (Eckert *et al*. 2019). A fragment of the mitochondrial gene, consisting of 843–859 base pairs encoding the mitochondrial *16S* rRNA gene, was amplified using the primers set L-2188/16H-1 (Hedges 1994, Matsui *et al*. 2006). Each PCR reaction contained 1X Myfi Reaction Buffer, 50 ng DNA template, 0.6 µM of each primer, and 2U of Polymerase (MyFi™ DNA Polymerase kit, Meridian Bioscience). The PCR reactions were conducted on the gradient thermocycler (Mastercycler® nexus, Eppendorf) under the following condition for the L-2188/16H-1 primer set: 95ºC for 1 min, followed by 30 cycles of 95°C for 15s, 60.5˚C for 30s, and 72°C for 30s, with a final extension step at 72°C for 5 min. A negative control was run simultaneously. All PCR setups and DNA extractions were performed in a clean room using a BioHazard Safety Cabinet (Daihan Labtech, Indonesia).

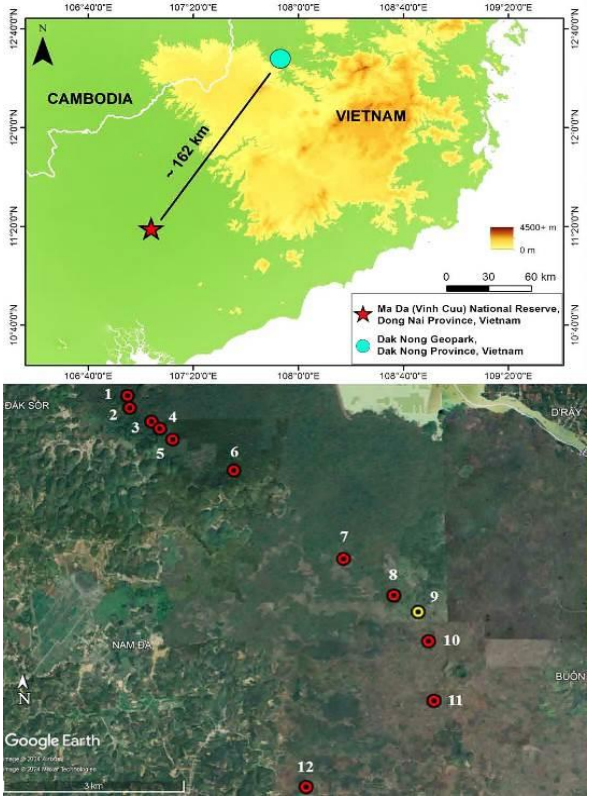

Figure 1. The distribution map of *Micryletta erythropoda* in Vietnam (above) and the map of surveyed lava caves (below) with caves, with *M. erythropoda* present (yellow circle) and absent (red circles); the numbers 1 to 12 represent caves C0, C1–C2, C3, C4, C6, C7, P20, P8, C8, P4, P6, and PT06.

The amplified products were visualized on 1% agarose gels, purified using an Isolate II PCR and Gel Kit (Bioline), and then Sanger sequenced (DNA SEQUENCING, Vietnam). The newly obtained *16S* nucleotide sequences were analysed with Chromatogram (Chromas software version 2.6.6) and combined with all available sequences of *Micryletta* species (Poyarkov *et al*. 2021, Sankar *et al*. 2022) (Sup. Table 1). The sequence dataset was aligned using MUSCLE (Edgar 2004) with default parameters in MEGA11 (Tamura *et al*. 2021). Uncorrected

p-distances were calculated using MEGA11 with default settings. *Kaloula pulchra*, *Mysticellus franki*, and *Uperodon systoma* were selected as outgroups according to Poyarkov *et al*. (2021). Phylogenetic trees were constructed using Bayesian Inference (BI) and Maximum Likelihood (ML) approaches. The best-fit model of sequence evolution for BI and ML, GTR+I+G, was selected based on the Akaike Information Criterion via MrModeltest 2.3 (Nylander 2004).

The BI analysis was performed using MrBayes v3.2 (Ronquist *et al*. 2012) for 1,000,000 generations, with sampling every 100 generations. The runs were terminated when the average standard deviation reached 0.004387. The first 25% of the trees were discarded as burn-in before generating a 50% majority consensus tree. The ML analysis was conducted using the IQ-TREE web server (Nguyen *et al*. 2015, Trifinopoulos *et al*. 2016) with 1,000 bootstrap pseudoreplicates via the ultrafast bootstrap (UFB) approximation algorithm (Hoang *et al*. 2018). Phylogenetic tree nodes were considered well-supported at a Bayesian posterior probability (BPP) ≥ 0.95 and ML ultrafast bootstrap support (UFB) ≥ 95% (Hillis & Bull 1993, Minh *et al*. 2013). FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree) was used to visualize the trees.

Morphological study. Measurements of the specimens were recorded to the nearest 0.1 mm using dial callipers. Paired meristic characters are presented as left/right from a dorsal view. We determined sex by the secondary sexual character, a single internal vocal sac in males but not in females in all *Micryletta* species (Poyarkov *et al*. 2018, Liu *et al*. 2021a). The morphometrics of adults and character terminology follow Poyarkov *et al*. (2018). The following measurements were used: SVL = snout-vent length; NSL = nostril-snout length, the distance between the middle of nostril and snout tip; HL = head length, from the tip of snout to hind border of jaw angle; SL = snout length, from the anterior corner of eye to the tip of snout; EL = eye length, distance between anterior and posterior corners of the eye; NEL = nostril-eye length, distance between the anterior corner of the eye and the nostril centre; HW = head width, maximum width of head at the level of mouth angles in ventral view; IND = internarial distance, distance between the central points of nostrils; IOD = interorbital distance, shortest distance between the medial edges of eyeballs in dorsal view; UEW = upper eyelid width, maximum distance between the medial edge of eyeball and the lateral edge of upper eyelid; TYL = tympanum length, horizontal tympanum diameter; FLL = forelimb length, length of straightened forelimb to the tip of third finger; LHL = lower arm and hand length, distance between elbow and the tip of third finger; HAL = hand length, distance between the proximal end of outer palmar (metacarpal) tubercle and the tip of third finger; FFL = first finger length, distance between the tip and the distal end of inner palmar tubercle; IPL = inner palmar tubercle length, maximum distance between proximal and distal ends of inner palmar tubercle; MPL = medial palmar tubercle length, measured as the maximum distance between proximal and distal ends of median palmar tubercle; OPL = outer palmar tubercle length, maximum distance between proximal and distal ends of outer palmar tubercle; 3FDD = third finger disk diameter; HLL = hindlimb length, length of straightened hindlimb from groin to the tip of fourth toe; TBL = tibia length, distance between the knee and tibiotarsal articulation; FL = foot length, distance between the distal end of tibia and the tip of fourth toe; OMTL = outer metatarsal tubercle length, maximum length of outer metatarsal tubercle; IMTL = inner metatarsal tubercle length, maximum length of inner metatarsal tubercle; 1TOEL = first toe length, distance between the distal end of inner metatarsal tubercle and the tip of first toe; 4TDD = fourth toe disc diameter.

Results

Phylogenetic analyses. The ML and BI analyses of the *16S* rRNA sequences recovered trees with similar topologies (Fig. 2). The sequences from the specimens collected in lava caves clustered closely with those of *M. erythropoda* from the Ma Da (Vinh Cuu) Nature Reserve in Dong Nai Province, Vietnam, showing strong support (BPP = 1.00; UFB = 100%). In terms of uncorrected pairwise distance (p-distance) (Sup. Table 2), the lava cave specimens exhibited the smallest genetic distance from *M. erythropoda* at 0.3%, compared to the population from the Dong Nai type locality. The genetic distance from *M. erythropoda* to its closest taxon, *M. lineata*, was calculated to be 2.2% from the lava cave specimens in Dak Nong UNESCO Global Geopark, and to be 2.5% from the specimens in Dong Nai Province. Notably, the genetic distance among specimens from lava cave C8, Dak Nong UNESCO Global Geopark was found to be 0.0%.

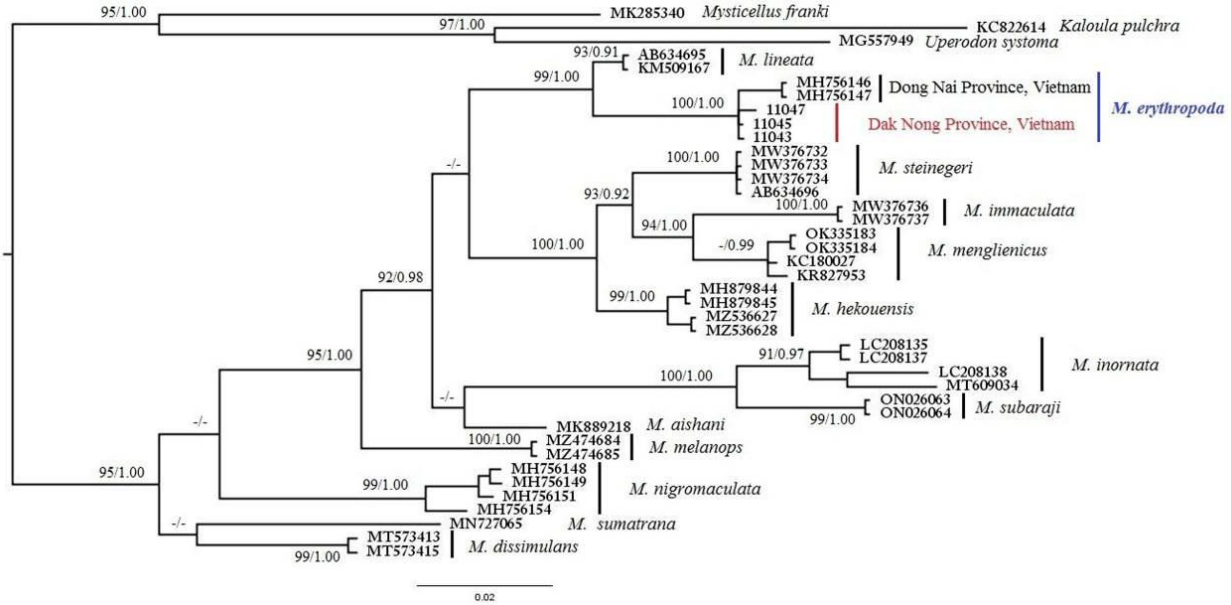

Figure 2. Bayesian inference (BI) tree for the *M. erythropoda* based on COI. Numbers left and right are ML ultrafast bootstrap (UFB) and Bayesian posterior probabilities (BPP), respectively; -/- = bootstrap values <90%.

Taxonomy

Micryletta erythropoda (Tarkhnishvili, 1994)
(Fig. 3; Sup. Table 3)

Specimens examined. Two adult males (ITBCZ 10224–10225) and one adult female (ITBCZ 10226), collected from lava cave C8, Dak Nong UNESCO Global Geopark, Krong No District, Dak Nong Province, Vietnam (12°30'32.5"N, 107°54'35.1"E) by V.H.D. Nguyen on 12 December 2020. Additional specimens collected from the same location on 20 October 2023 by V.H.D, Nguyen, and T.G. Tran include three adult females (ITBCZ 11043–11045), one subadult female (ITBCZ 11047), and two adult males (ITBCZ 11046, 11048). All specimens and liver tissue samples are deposited in the Institute of Tropical Biology Collection of Zoology (ITBCZ), District 3, Ho Chi Minh City, Vietnam.

Description of the male. An adult male specimen (ITBCZ 11046). Body small-sized (SVL 22.5 mm), habitus moderately slender, elongated, and oval shaped; head wider than long (HL/HW 0.88); snout short (SL/SVL 0.14) rounded dorsally, slightly acuminate in profile, and projecting beyond margin of lower jaw; eye comparatively large (EL/HL 0.33), lightly protuberant, shorter than snout length (EL/SL 0.68), larger than interorbital (EL/IND 1.24). Top of head flat, canthus rostralis rounded and distinct; loreal region slightly concave; nostril oval, lateral opening, closer to snout than to eye (NSL/NEL 0.53); interorbital distance wider than internarial distance (IOD/IND 1.59), approximately two times wider than upper eyelid (IOD/UEW 2.25); tympanum small (TYL/HL 0.17), rounded, distinct; supratympanic fold very indistinct. Tongue oval; choanae elongated and oval-shaped, widely spaced; vomerine teeth absent; tongue without papillae, spatulate, lacking posterior notch and free for ½ from floor of mouth.

Forelimbs slender, short (FLL/SVL 0.74), hand length shorter than forelimb length (HAL/FLL 0.38). Fingers slender, completely free of webbing, and lacking lateral skin fringes; relative finger lengths: I<II<VI<III; tips of all fingers rounded, and not expanded to disks; subarticular tubercles on fingers distinct, rounded, prominent, and subarticular tubercle formula: 1, 2, 3, 3; supernumerary tubercles on palm present and well-developed; three metacarpal tubercles: inner one distinct, oval-shaped, smallest (0.4 mm in diameter): median one large, prominent (0.6 mm in diameter), separated to outer metacarpal tubercle: outer one largest, oval (0.7 mm in diameter); nuptial pad absent.

Hindlimbs slender and longer than snout-vent length (HLL/SVL 1.53), more than two times length of forelimb (HLL/FLL 2.08); tibia long (TBL/SVL 0.50) and slender, approximately one-third of hindlimb length (TBL/HLL 0.32); tibiotarsal articulation of adpressed limb reaching middle of eye. Toes long and slender, relative toe lengths: I<II<V<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 0.80); rudimentary web between toes II–III and III–IV; 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 three times of first toe (IMTL/1TOEL 0.29); outer metatarsal tubercle round shaped, smaller than inner one (OMTL/IMTL 0.33); two small supernumerary tubercles at base of toes II and III, smaller than proximal subarticular tubercles.

Dorsal skin smooth above, posteriorly scattered with tiny and flat tubercles on dorsum of body, flanks, and hindlimbs; subtle longitudinal median ridge present on dorsum (visible in life; nearly invisible in preservation); lateral sides of head smooth; dorsolateral fold absent; ventral skin of body and limbs smooth.

Colouration. In life (Fig. 3), dorsum brick red with 22 black blotches. Flank less red than colour on dorsum on upper part and white on lower part, large black blotches present; a black patch running from snout to flank with white blotches present below the eye and tympanum areas. Dorsal surfaces of forelimbs and hindlimbs brick red to white with black blotches but the red part from armpit to elbow is nearly entire without mottling. Ventral surfaces cream with small and irregular white marbling patterns but dense on chest and belly. Throat notably darker, margins of lower jaw with distinct irregular white marbling. Pupil round; iris bicolored, with upper third golden and lower two-thirds dark brown. In preservative: Colours greatly faded; ventral side dull white with light gray marbling on chest and lateral sides of belly; chin distinctly greyish black.

Variation in cave specimens. In contrast to the mainly brick red coloration dorsally, one specimen (ITBCZ 11048) has gold coloration. Throat with or without dark pigmentation. Tibiotarsal articulation of adpressed limb reaching posterior edge of tympanum or middle of eye. Median and outer metacarpal tubercles separated or fused partly (Fig. 3).

Natural history. On 21 October 2023, at approximately 15:30, we encountered individuals of *M. erythropoda* approximately 140 meters inside lava cave C8, located in an agricultural area (Fig. 4). The surrounding area is primarily used for seasonal corn cultivation, a practice dating back centuries. The frogs were found resting in depressions and cavities in the rock walls, around 20 meters below ground level. The temperature at this depth was approximately 26.5 °C, with a humidity level of approximately 92.5%. These conditions were notably cooler and more humid than at the cave entrance, where the temperature was 28.8 °C and the humidity was 79.6%. The only light source in this area came from a hole, approximately one meter in diameter, in the cave ceiling, approximately 15 meters above the floor of the cave. However, the encounter occurred in darkness, with a light intensity of approximately 0.0 lux.

Discussion

Our specimens matched the description provided by Tarkhnishvili (1994) and Poyarkov *et al.* (2021). However, the population in lava cave C8, Dak Nong UNESCO Global Geopark, displayed some differences from the original description. These include the presence of a subtle longitudinal median ridge present on dorsum in life; tibiotarsal articulation of adpressed limb reaching from posterior tympanum to middle of eye; and metacarpal tubercles on palm that are either partly fused or separated.

Notable sexual dimorphism: females larger than males (SVL 26.3±1.7 mm in four females vs. 21.6±0.9 mm in four males); chin notably darker in all males; median and outer metacarpal tubercles separated in male vs. partly fused in females, except for specimen ITBCZ 11045, which is separated) (Sup. Table 3).

Our observation confirms the distribution of *M. erythropoda* in Dak Nong Province, Vietnam, at approximately 162 km from the type locality (Tarkhnishvili 1994). This finding marks the first documented case of cavernicolous behaviour not only for this species but also for the entire genus *Micryletta*. However, the habitat within cave C8 (one of the caves with an entrance situated in a large, extensive seasonal agricultural monoculture) is facing threats from human activities. These threats include deforestation, plastic pollution, and tourism. Consequently, enhancing our understanding of cave biodiversity, not just in cave C8 but throughout the entire lava cave ecosystem in Dak Nong UNESCO Global Geopark, along with cave ecology and interactions with the environment, is essential for supporting the conservation of these fragile and unique habitats.

Currently, our knowledge of *M. erythropoda* is primarily focused on taxonomy, with several studies on related species (*e.g*., Poyarkov *et al*. 2018, 2021, Alhadi *et al*. 2019, Das *et al*. 2019, Munir *et al*. 2020, Suwannapoom *et al*. 2022, Liu *et al*. 2021a, Miller *et al*. 2021, Sankar *et al*.

Plate 26

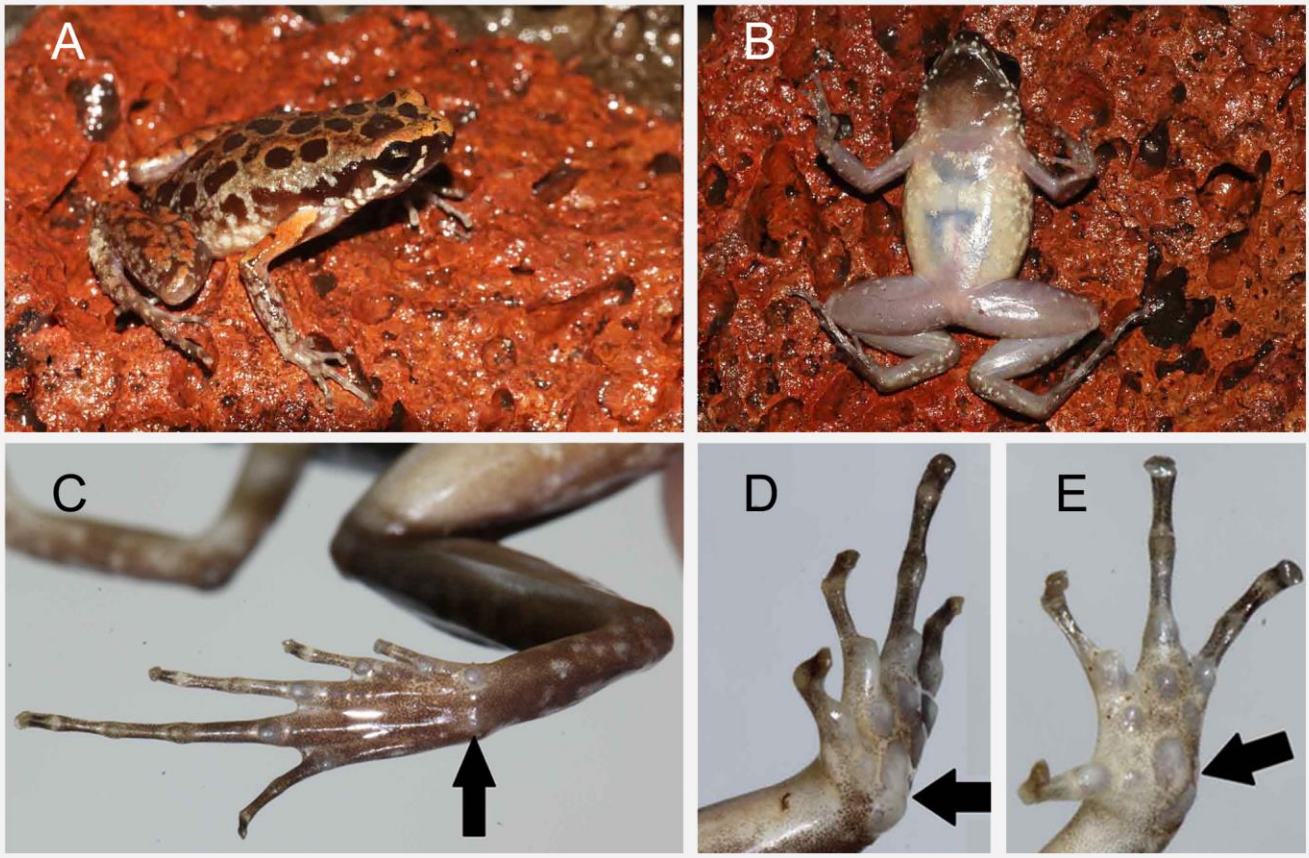

Figure 3. *Micryletta erythropoda* in crevices on the lava rock walls inside cave C8 (ITBCZ 11046): **(A)** dorsolateral and **(B)** ventral views of the full body; **(C)** outer metatarsal tubercle; median and outer meta carpal tubercles **(D)** fused or **(E)** separated.

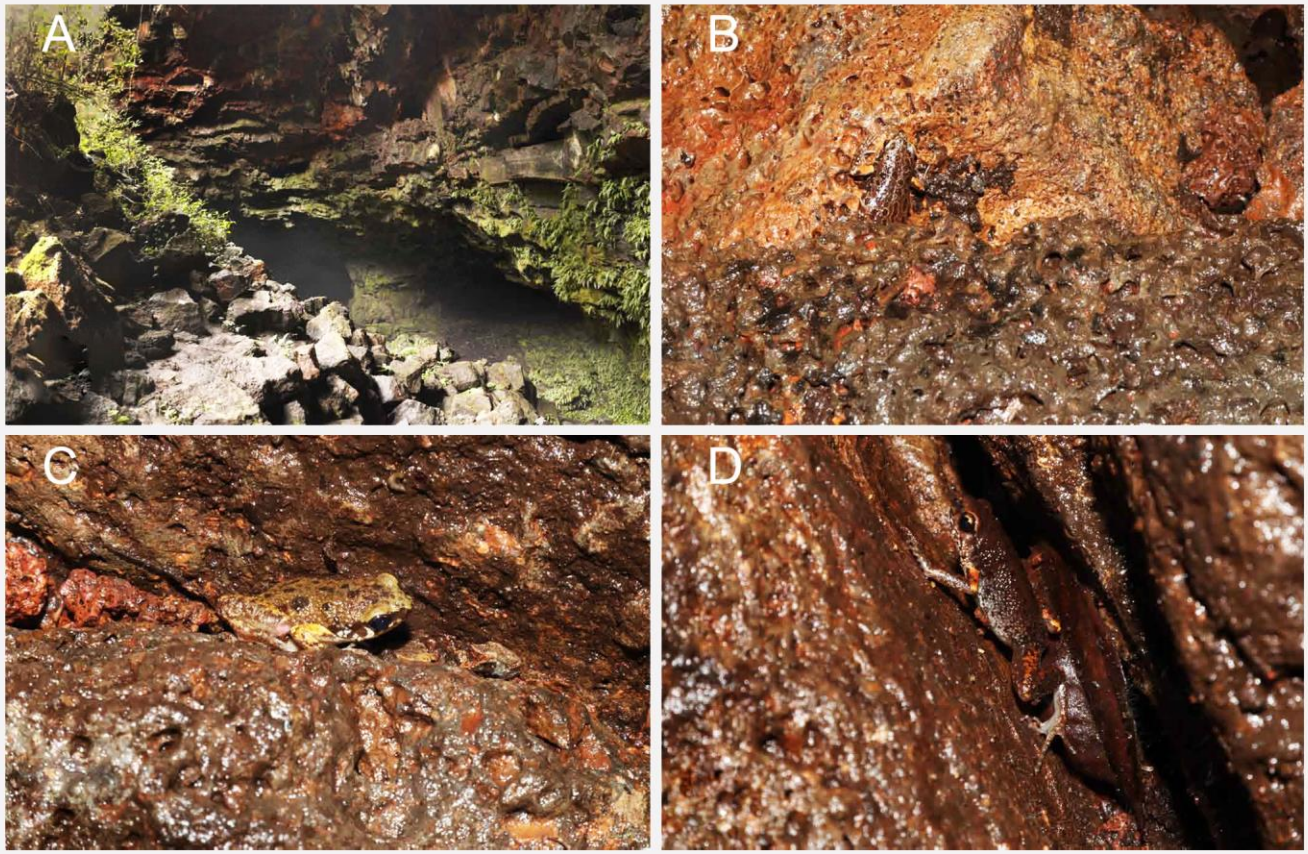

Figure 4. (A) Cave habitat and **(B-D)** microhabitat of *Micryletta erythropoda* inside cave C8

2022, Nguyen *et al*. 2024), while other aspects remain largely unexplored (Thongproh *et al*. 2018). Further study is needed in several areas, as it is for other anurans and animal groups, including buccopharyngeal features (Chunskul *et al*. 2021), predator and prey interactions (Moonasa *et al*. 2018, Dowwiangkan *et al*. 2020, Thongproh *et al*. 2019, 2020), habitat suitability (Phommexay *et al*. 2024a), population dynamics and threats (Dowwiangkan *et al*. 2018, Phommexay *et al*. 2024b), and impacts of environmental pollution (Chuaynkern *et al*. 2024). Our understanding of the biology and ecology of *M. erythropoda* in lava caves remains limited, underscoring the need for urgent research. Key questions include: Why does this population inhabit subterranean environments? How do they select and adapt to these habitats? Do they feed within the cave and, if so, what prey do they consume? At what stage of life do they begin using the cave? Lastly, does reproduction occur within this cave environment?

Author contributions

VDHN (principal investigator) contributed to conceptualization, methodology, project administration, data curation, formal analysis, software, validation results, and writing; TGT contributed to data curation, formal analysis, methodology, software, and writing; ML, MAN, HDH, TTPN, Nguyen improved the draft manuscript.

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Supplement data

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