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New record and dietary ecology of a poorly known amphibian species, *Micryletta menglienica* (Yang and Su, 1980), from Vietnam

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Running title: Dietary ecology of an endangered amphibian species

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Abstract. Recent field surveys revealed a new population of the Menglian Paddy Frog (*Micryletta menglienica*) from Son La Province, northwestern Vietnam based on molecular and morphological analyses. The species has been listed as "Data Deficient" in the IUCN Red List. To date, knowledge about the natural history of this species is scarce, including data on its dietary ecology. Using the stomach-flushing method, we analyzed stomach contents of 45 individuals (21 males and 24 females) from a total of 52 captured frogs because stomachs of six males and one female (13.5%) were empty. The food spectrum of *M. menglienica* comprises 12 types of 691 prey items (687 invertebrate items and four unidentified items), belonging to six insect orders, larvae and Araneae. Similar to other species of the family Microhylidae, *M. menglienica* is also identified as an ant (Formicidae) – termite (Rhinotermitidae) eating specialist with distinctly higher percentages of occurrence frequency ($F = 65\%$), number of prey items ($N = 80\%$), and importance ($I_x = 75\%$) than those of remaining types. The Simpson diversity index of 0.939 shows a high abundance of food prey in the wild, and the Evenness index of 0.536 suggests biased consumption of feeding selection for *M. menglienica*.

Keywords. Formicidae, Prey items, Rhinotermitidae, Son La Province, Stomach contents.

INTRODUCTION

Inhabiting both freshwater and terrestrial ecosystems, amphibians play a paramount role in maintaining the stability of food webs and energy flow between their habitats (Duellman and Trueb, 1994; Wells, 2007). In particular, amphibians transfer the energy intake by consuming many invertebrates, even small vertebrate species, to higher trophic levels by serving as an essential food source for several predatory animal groups (such as reptiles and mammals), accounting for a major part of animal biomass in ecosystems (Burton and Likens, 1975; Toledo et al., 2007; Oliveira et al., 2013). Thus, understanding the trophic niche may provide additional insights into species' natural history and biotic interactions (Schoener, 1974; Toft, 1980; Prado et al., 2005; Wells, 2007; Duré et al., 2009). To investigate this matter in amphibians, the stomach content is often obtained to identify which prey was eaten and their relative importance in the species' diet (Maneyro et al., 2004; Caldart et al., 2012; Le et al., 2018). In terms of conservation assessments, these analyses provide crucial data on conditions and resources required for species survival under human impacts (e.g., habitat loss, degradation, and overexploitation for trade and food consumption) that can imperil the stability in animal communities with intermittent trophic chains (Clavel et al., 2011). However, detailed information on the diet niche of many amphibian species is still lacking.

The paddy frogs of the genus *Micryletta* Dubois 1987 currently consist of 13 species, distributed from northeastern India and China through the Indochina region and expanding southward to west Malaysia and Sumatra (Frost, 2024). Remarkably, as many as eight species of *Micryletta* have been discovered in the last five years (Frost, 2024). The Menglian Paddy Frog, *Micryletta menglienica* (Yang and Su, 1980) was originally described based on type series collected in Menglien, Yunnan Province, southern China (Yang and Su, 1980; Liu et al., 2021) and subsequently recorded from Lang Son and Cao Bang provinces, northern Vietnam (Ohler and Grosjean, 2005). It has recently been reassigned to the genus *Micryletta* after having been

placed in the genus *Kalophrynus* (Fei, 2020; Liu et al., 2021). The species is currently classified as Data Deficient in the IUCN Red List because little data on its population status and natural history are available (IUCN, 2023).

As a result of our field surveys in Son La Province, northwestern Vietnam, individuals of *Micryletta* were collected and their morphological characteristics closely resemble those of *Micryletta menglienica* as reported by Liu et al. (2021). We herein confirm the record of the species for the first time from Son La Province based on analyses of molecular and morphological data. In addition, to better understand the natural history of dietary ecology of the poorly known species, we analyzed the stomach content of 52 captured individuals to identify prey items and determined their relative importance in the species' diet.

MATERIALS AND METHODS

Field surveys and sampling

Field surveys were conducted in June, July, and September 2017 in two localities of Son La Province, northwestern Vietnam: the first site in Son La City (21°18.659'N, 103°46.956'E, at an elevation of 550 m) and the second one in Phong Lai Commune, Thuan Chau District (21°36.458'N, 103°33.980'E, at an elevation of 680 m) (Table 1, Fig. 1). The survey transects were set up with lengths ranging from 2.0 to 3.0 km, in limestone karst forests and open areas of grass and shrubs. We captured frogs by hand between 20:00 and 24:00h and used a stomach-flushing technique to obtain stomach contents without sacrificing them (Griffiths, 1986; Leclerc and Courtois, 1993; Solé et al. 2005; Norval et al., 2014). Prey items were preserved in 70% ethanol and deposited at the Faculty Environmental of Science, University of Science, Vietnam National University, Hanoi, Vietnam. Frogs were subsequently released at the collecting site after measurements of snout-vent length (SVL) and mouth width (MW) with a digital caliper

to the nearest 0.01 mm taken. In total, 52 frogs, including 27 males and 25 females, were caught at two sites (table 2).

Species identification

For taxonomic identification, four individuals were collected for voucher specimens. After having been photographed in life, animals were anesthetized and euthanized in a closed vessel with a piece of cotton wool containing ethyl acetate (Simmons, 2002), fixed in 85% ethanol and subsequently stored in 70% ethanol. Determination of species was based on morphology following Liu et al. (2021). We also sequenced two new samples of *Micryletta menglienica* collected from Son La Province. We used the protocols of Le et al. (2006) for DNA extraction, amplification, and sequencing. A fragment of 16S rRNA gene, approximately 480bp was amplified and sequenced using a primer pair 16Sar and 16Sbr (Palumbi et al. 1991). Sequences were compared with those available from GenBank using Basic Local Alignment Search Tool (BLAST) searches.

Stomach content analysis

In the laboratory, prey items were identified under microscopes (Olympus SZ 700) following taxonomic literature of invertebrates (i.e., Naumann et al., 1991; Johnson and Triplehorn, 2005). The maximum length (L) and width (W) of each prey item were measured to the nearest 0.01 mm using either a digital caliper or a calibrated ocular micrometer fitted to a microscope (Hirai and Matsui, 2001). The volume (V, mm³) of prey items was calculated using the formula for a prolate spheroid ($\pi = 3.14$; Magnusson et al., 2003): $V = 4\pi/3 \times (L/2) \times (W/2)^2$. The index of relative importance (Ix), was used to determine the importance of each food type, which was calculated following the formula: $Ix = (\%F + \%N + \%V)/3$ (Caldart et al., 2012), where %F (F – Frequency of occurrence) is a percentage of stomach containing each prey type, %N (N – Number) is a percentage of number of each prey item in all.

We used the reciprocal Simpson's heterogeneity index, $1-D$, to calculate dietary heterogeneity: $D = \sum [n_i(n_i - 1)] / [N(N - 1)]$; where n_i is the number of food items in the i^{th} taxon category and N is the total number of prey items (Krebs, 1999). We used another index to estimate prey evenness. The evenness index is calculated from the equation: $J' = H' / H_{\max} = H' / \ln S$; where S is the total number of prey taxa and H' is the index of taxon diversity. The value of H' is calculated from the equation: $H' = -\sum (p_i \times \ln p_i)$; where p_i is the proportion of food items belonging to the i^{th} taxon for the total food items of the sample (Magurran, 2004; Muñoz-Pedreros and Merino, 2014).

Two-way ANOVA tests were performed to determine differences or similarities in morphological characteristics of the species, sizes, and volume of ingested food between males and females, between populations, and the combined effects of "Sex + Location". Post hoc Tukey tests (TukeyHSD) were afterwards applied to check the difference between all pairs of sex groups in each population, and location groups in each sex after the ANOVA tests. Females were identified by having longer snout-vent length ($SVL \geq 21.0$ mm), whereas males were characterized by a single internal vocal sac and shorter SVL (< 21.0 mm). All statistical analyses were performed by using the software environment R.3.1.2 (RStudio Team, 2018).

RESULTS

A new record of Micryletta menglienica in Son La Province

Pham et al. (2016) reported *Micryletta inornata* (Boulenger, 1890) from Co Ma Commune, Thuan Chau District, Son La Province, Vietnam. However, the specimens were re-identified herein as *M. menglienica* because two sequences (GenBank accession numbers PQ634813 and PQ634814) obtained from the collected samples in Son La Province were identical to each other and 99.35% similar to those with accession numbers OK335186 and OK335187 of *M.*

menglienica available on GenBank. This is also the first record of *M. menglienica* from Son La Province, northwestern Vietnam.

Morphological characteristics of paddy frogs collected in Son La Province match well with the diagnosis of *Micryletta menglienica* (Liu et al., 2021): size small (SVL: 14.8–27.7 mm, n = 52); head wider than long; snout obtusely round in profile; the interorbital distance wider than upper eyelid; tympanum indistinct; supratympanic fold distinct; vomerine teeth absent; tibiotarsal articulation of adpressed limb reaching the level of eye; dorsal surface granular with small round tubercles; subtle longitudinal median ridge present on dorsum; supratympanic fold present; dorsolateral fold absent; outer metatarsal tubercle absent; dorsum coloration purple brown, blueish gray or dark brown; body flanks brown with dark-brown to black patches or spots edged with white, a large black streak extending on each side from tip of the snout to crotch; ventral surface greyish brown, whitish with light-gray marbling patterns on chest and belly (Fig. 2).

Regarding sexual dimorphism, males have a shorter snout-vent length (SVL: 19.03 ± 1.95 mm, ranging 14.8 – 20.8 mm, n = 27; ANOVA-test, $F = 124.2$, $P\text{-value} < 0.001$) and a narrower mouth width (MW: 5.6 ± 0.73 mm, ranging 4.4 – 6.7 mm, n = 27; $F = 52.1$, $P\text{-value} < 0.001$) than those of females (SVL: 24.73 ± 1.4 mm, ranging 21.5 – 27.7 mm and MW: 6.77 ± 0.49 mm, ranging 6.0 – 7.8 mm, n = 25, respectively). Together with the effect of locations, the tests did not approve the general difference in both snout-vent length and head width ($P\text{-values} > 0.05$). In particular, the intra-sexual variations in both morphological traits of *M. menglienica* were not presented between two populations of Son La City and Phong Lai Commune (Tukey's HSD tests, $P\text{-values} < 0.001$), whereas males' traits were significantly different to females', noted in each population (Tukey's HSD tests, $P\text{-values} < 0.001$).

In terms of natural history, paddy frogs were found between 19:00 and 24:00 at elevations between 550 and 680 m a.s.l. The surrounding habitat was the limestone karst forest of small

hardwoods, shrubs and grasses (Fig. 2). The relative humidity was approximately 70 – 85% and the air temperature ranged from 25 to 30°C. Other sympatric amphibian species were also observed, including *Leptobranchella* sp., *Microhyla butleri* Boulenger 1900, *M. heymonsi* Vogt 1911, and *Polypedates megacephalus* Hallowell 1861.

Dietary ecology

A total of 52 individuals (27 males and 25 females) of *M. menglienica* were captured in Son La City (17 individuals) and Thuan Chau District (35 individuals) of Son La Province. All caught individuals were checked for stomach contents, except for seven frogs (13.5%), whose stomachs were empty. Based on the obtained contents, we identified a total of 691 ingested food items (687 invertebrates and four unidentified items) belonging to 12 different prey types of six insect orders: Blattodea, Coleoptera (Coccinellidae, Staphylinidae, Tenebrionidae, and Other Coleoptera), Diptera (Syrphidae, Other Diptera), Hymenoptera (Formicidae), Isoptera (Rhinotermitidae), and Orthoptera (Gryllidae), Insect larvae, and Spiders (Araneae).

In terms of size measurements, the item-mean width (Wm) of ingested prey was 1.33 ± 0.12 mm (0.2 – 5.0 mm), the item-total width (Wt) was 15.7 ± 2.56 (0.5 – 66.0 mm), the item-mean length (Lm) was 2.8 ± 0.19 mm (0.64 – 7.23 mm), and the item-total length (Lt) was 35.24 ± 6.6 (2.0 – 184.8 mm) (Fig. 3, Table 2). We calculated the mean volume of 44.02 ± 8.41 mm³ (0.26 – 224.6 mm³). There was only a significant difference in the item-mean width (Wm) of consumed prey between males and females, and the interaction of “Sex + Locality” (Two-way ANOVA tests, P-values = 0.04 < 0.05), whereas remaining prey characteristics (i.e., Wt, Lm, Lt and volume) were relatively similar between intraspecific sexes, localities and combined effect of “sex + locality” (P-values > 0.05; Fig. 3, Table 2). Our multiple linear regression analyses showed that the sizes and volume of ingested prey are not significantly correlated with morphological traits of SVL and MW of the species (table 2).

The prey of *M. menglienica* mainly comprised two invertebrate families of Formicidae and

Rhinotermitidae, which accounted for more than 65% of the frequency of occurrence (F) and 80% of the number of prey items (N) (Table 3). The two invertebrate families were also the most important prey of the species, accounting for more than 75% of Ix (Table 3).

The dietary breadth of *M. menglienica* from Son La Province, expressed by the Simpson's index of diversity was 0.939, and evenness was 0.536 (Table 4). Males of *M. menglienica* had higher values of both Simpson index (0.926) and evenness (0.783) than those of females (Table 4). The population of *M. menglienica* in Son La City consumed less diverse prey than the population in Thuan Chau District (Table 4).

DISCUSSION

In this study, *M. menglienica* was recorded for the first time in Son La Province, which is approximately 350 km from the type locality of the species in Puer City, Yunnan Province, China. With the new record of *M. menglienica* in Son La Province, we recommend additional field surveys be conducted in other karst ecosystems in northern Vietnam to look for new populations of the species and better understand the species distribution range.

A total of 12 different prey types belonging to six invertebrate orders, larvae and spiders (Araneae) were identified as the food spectrum of *M. menglienica*, in which ants (Formicidae) and termites (Rhinotermitidae) were determined as the most dominant and important prey of the species. Anurans, especially in the family Microhylidae, have been widely recognized as ant or sometimes termite specialists (Toft, 1980; Solé et al., 2002; Isacch and Barg, 2002; Berazategui et al., 2007; Atencia et al., 2017; Lopes et al., 2017).

In this study, we did not find any intersexual difference in the sizes and volume of prey eaten by *M. menglienica*. Competitive interactions among conspecifics for sharing resources could take place between males and females (Schoener, 1974; Irschick et al., 2005; van Schingen et al., 2015). Our estimation based on the number of food items for indices of Simpson diversity

(more than 0.88) showed a high abundance of prey at the studied sites, but not evenly among food types (0.536 – Evenness index). Furthermore, females' prey (Evenness index = 0.5) was more biased than those in males (0.783). All of these can reduce intersexual competition. Pham et al. (2022) studied the diet niche of *Microhyla butleri* and *M. heymonsi* in Son La Province and showed that both sympatric species primarily consumed ants and termites as well. However, there were significant differences in the trophic niche between them to limit interspecific competition. In particular, 11 prey types of *M. butleri* were not found in the food spectrum of *M. heymonsi* and nine prey types were not presented in vice versa. Furthermore, they preferred to consume significantly different prey in the sizes and volume (Pham et al. 2022).

Regarding human impacts, karstic forest degradation caused by infrastructure development (e.g., road expansion, development of residential areas, and limestone quarrying) has been documented in Son La City (Pham pers. obs), which could explain the low number of *M. menglienica*. Only 17 individuals of *M. menglienica* were captured in Son La City during three surveys, compared to half of captured animals ($n = 35$) in less disturbed habitats in Phong Lai Commune. Such human impacts particularly destabilize its wild populations and food availability. However, similar to what is happening in Son La Province, unsustainable human activities (particularly quarrying and tourist activities) over karst ecosystems in northern Vietnam, can extensively impact undiscovered populations of the species in unique karstic habitats (Clemens et al. 2006). Therefore, conservation measures are needed to protect the species, though *M. menglienica* has not been targeted for the pet trade and food consumption, and its geographical distribution is potentially extended. However, to date, no conservation plan has been developed to safeguard the poorly known species as well as its natural habitats. Besides the investigation of dietary ecology, we highly recommend further studies to identify the entire geographical extent, assess population status, and determine other ecological traits of

M. menglienica. Only when the background data becomes available, appropriate conservation measures can be designed to better safeguard the species from current extinction risks.

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Tables.

Table 1. The number of paddy frogs were captured from Son La Province, northwestern Vietnam.

Locality	Time	Number of individuals		
		Male	Female	Total
Son La City	10-13 June 2017	5	3	8
	6-9 July 2017	3	4	7
	5-7 September 2017	2	0	2
Phong Lai	16-19 June 2017	6	9	15
Commune	15-18 July 2017	8	6	14
	11-13 September 2017	3	3	6
Total		27	25	52

Table 2. Sizes and volume of prey consumed by *Micryletta menglienica* in relation to morphological characteristics (SVL: snout-vent length, MW: mouth width, Wm: item-mean width, Wt: item-total width, Lm: item-mean length, and Lt: item-total length (Lt) of prey items; and asterisks (*) indicating the significant difference with the P-value < 0.05).

	Total	Female	Male	Test (Sex)	Test (Locality)	Test (Sex + Locality)	SVL	MW
Wm (mm)	1.33 ± 0.12 (0.2 – 5.0)	1.11 ± 0.09 (0.5 – 2.67)	1.58 ± 0.23 (0.2 – 5.0)	F = 4.4; P = 0.04 < 0.05*	F = 3.05; P = 0.09 > 0.05	F = 4.3; P = 0.04 < 0.05*	r = -0.18; P = 0.25 > 0.05	r = 0.02; P = 0.92 > 0.05
Wt (mm)	15.7 ± 2.56 (0.5 – 66.0)	18.5 ± 4.28 (0.5 – 66.0)	12.5 ± 2.4 (1.0 – 50.7)	F = 1.4; P = 0.24 > 0.05	F = 1.8; P = 0.19 > 0.05	F = 0.9; P = 0.3 > 0.05	r = 0.14; P = 0.34 > 0.05	r = 0.05; P = 0.72 > 0.05
Lm (mm)	2.8 ± 0.19 (0.64 – 7.23)	2.81 ± 0.25 (1.07 – 7.23)	2.78 ± 0.31 (0.64 – 5.8)	F = 0.008; P = 0.9 > 0.05	F = 0.01; P = 0.91 > 0.05	F = 0.4; P = 0.55 > 0.05	r = 0.06; P = 0.69 > 0.05	r = 0.24; P = 0.11 > 0.05
Lt (mm)	35.24 ± 6.6 (2.0 – 184.8)	46.32 ± 11.52 (2.0 – 184.8)	22.6 ± 4.01 (2.0 – 72.0)	F = 3.4; P = 0.07 > 0.05	F = 1.6; P = 0.2 > 0.05	F = 0.4; P = 0.54 > 0.05	r = 0.23; P = 0.14 > 0.05	r = 0.14; P = 0.35 > 0.05
Volume (mm ³)	44.02 ± 8.41 (0.26 – 224.6)	40.8 ± 10.9 (0.26 – 188.4)	47.7 ± 13.3 (0.3 – 224.8)	F = 0.16; P = 0.69 > 0.05	F = 0.27; P = 0.61 > 0.05	F = 1.9; P = 0.18 > 0.05	r = -0.06; P = 0.7 > 0.05	r = -0.02; P = 0.91 > 0.05

Table 3. Dietary composition of *Micryletta menglienica* in Son La Province: F = frequency of occurrence, N = number of items, V = volume (mm³), Ix = index of relative importance of each prey type (n = 45 stomach contents)

Prey type	F	%F	N	%N	V	%V	Ix
Araneae	1	1.41	1	0.14	1.57	0.08	0.54
Blattodea	1	1.41	1	0.14	20.12	1.02	0.86
Coleoptera							
Coccinellidae	2	2.82	2	0.29	9.42	0.48	1.19
Staphylinidae	3	4.23	3	0.43	4.31	0.22	1.63
Tenebrionidae	1	1.41	1	0.14	2.74	0.14	0.56
Other Coleoptera	6	8.45	9	1.30	219.35	11.07	6.94
Diptera							
Syrphidae	1	1.41	1	0.14	5.65	0.29	0.61
Other Diptera	1	1.41	3	0.43	36.24	1.83	1.22
Hymenoptera							
Formicidae	36	50.70	476	68.89	632.57	31.94	50.51
Isoptera							
Rhinotermitidae	11	15.49	91	13.17	901.84	45.53	24.73
Orthoptera							
Gryllidae	2	2.82	2	0.29	12.04	0.61	1.24
Insect larvae	2	2.82	97	14.04	14.26	0.72	5.86
Unidentified	4	5.63	4	0.58	120.67	6.09	4.10
Total	71	100	691	100	1980.77	100	100

Table 4. Prey diversity and evenness indices of *Micryletta menglienica*, expressed with value (95 % confidence limits).

	Simpson 1-D	Evenness
Species (<i>Micryletta menglienica</i>)	0.939 (0.930 – 0.9438)	0.536 (0.495 – 0.573)
Male	0.926 (0.909 – 0.933)	0.783 (0.684 – 0.825)
Female	0.8896 (0.877 – 0.899)	0.50 (0.471 – 0.562)
Son La City	0.761 (0.670 – 0.818)	0.566 (0.466 – 0.697)
Phong Lai Commune	0.9255 (0.9158 – 0.932)	0.5647 (0.516 – 0.599)

Legends of figures

Fig. 1. Map of surveyed sites in Son La Province, northwestern Vietnam: (1) Son La City and (2) Phong Lai Commune.

Fig. 2. Natural habitat and an individual of *Micryletta menglienica* A, C. in Phong Lai Commune, B, D. in Son La City.

Fig. 3. Size and volume of prey consumed by *Micryletta menglienica* in relation to snout-vent length (left column), to sex (middle column), and locality (right column) (Wm: item-mean width, Wt: item-total width, Lm: item-mean length, Lt: item-total length, and V: volume of prey items; and asterisks (*) indicating the significant difference with the P-value < 0.05).

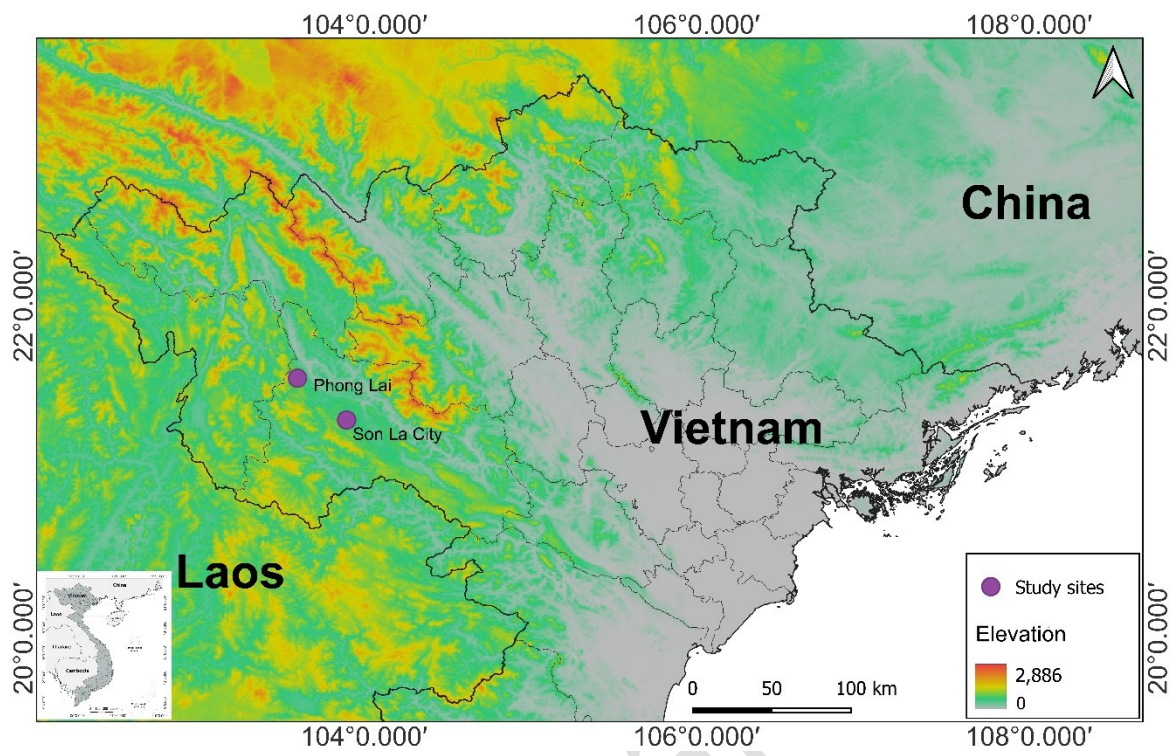


Fig. 1.

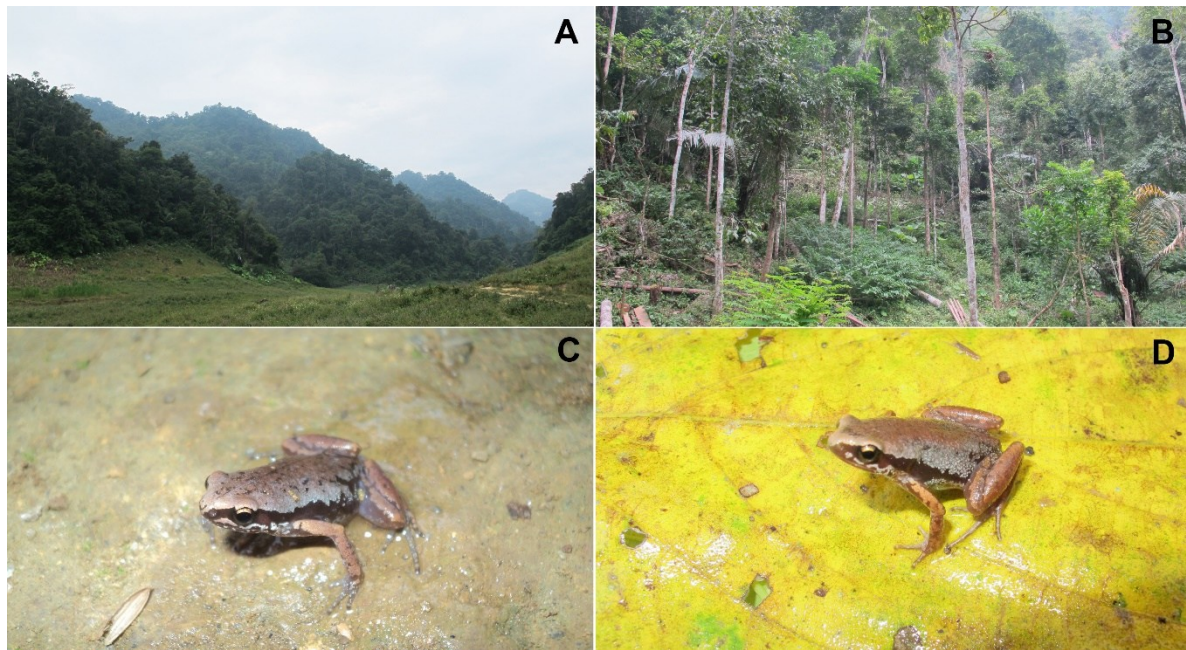


Fig. 2.

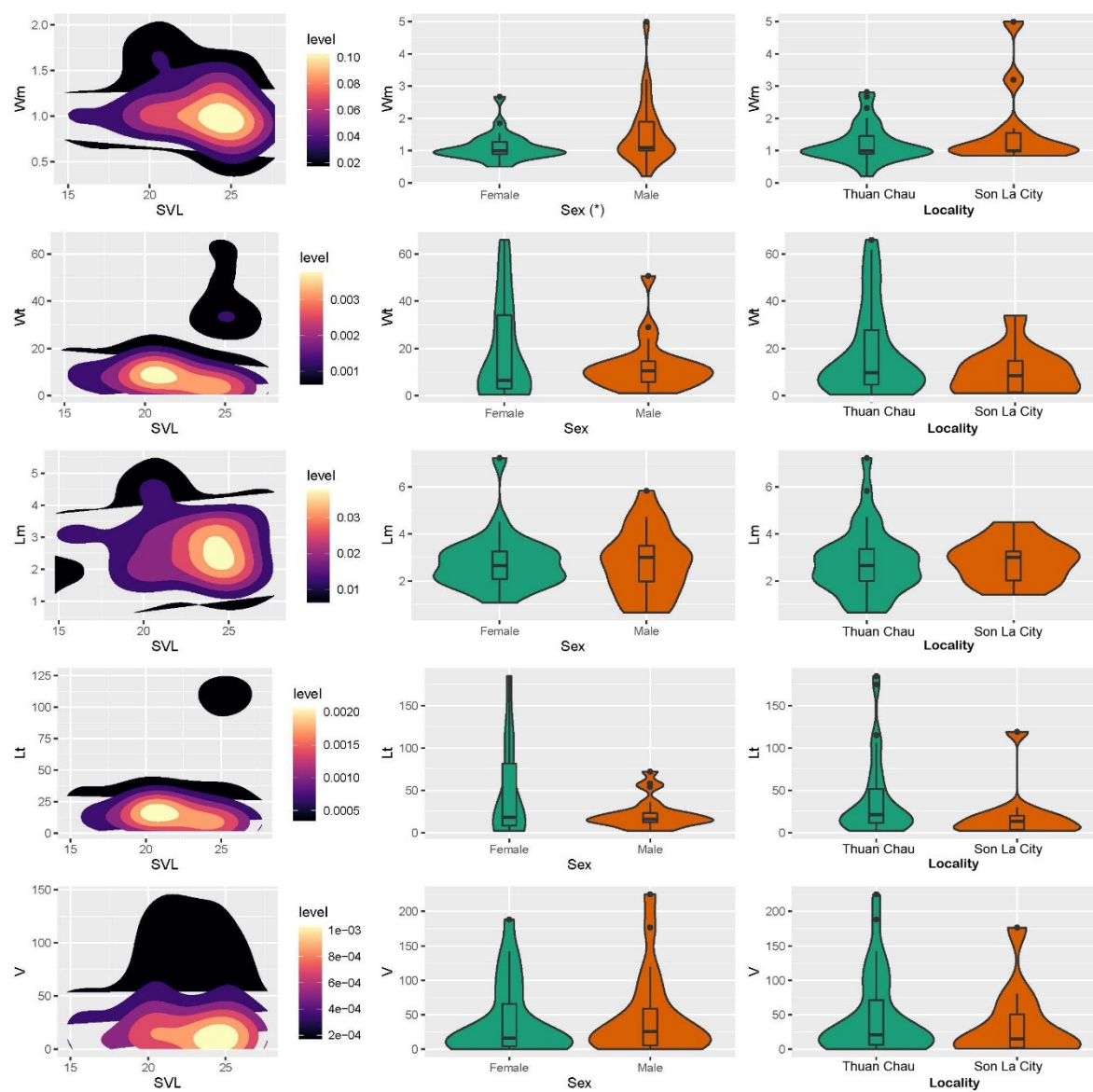


Fig. 3.