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New record and dietary ecology of an endangered amphibian species, *Micryletta nigromaculata* Poyarkov, Nguyen, Duong, Gorin & Yang, 2018, from Vietnam

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Abstract. The Black-spotted Paddy Frog (*Micryletta nigromaculata*) was recently discovered from northern Vietnam. Due to its restricted distribution in limestone karst forests and habitat degradation, the endemic species has been listed as "Endangered" in the IUCN Red List. However, knowledge about the natural history of this species is scarce, including data on its dietary ecology. Recent field surveys revealed new populations of *M. nigromaculata* occurring in Son La Province, northwestern Vietnam based on morphological analysis. 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. nigromaculata* comprises 12 types with a total 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. nigromaculata* 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 (Ix = 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. nigromaculata*.

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

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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 and 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, 2023). Remarkably, as many as eight species of *Micryletta* have been discovered in the last five years (Frost, 2023). The Black-spotted Paddy Frog, *Micryletta nigromaculata* Poyarkov, Nguyen, Duong, Gorin & Yang 2018, was originally described in Hai Phong and Ninh Binh provinces, northern Vietnam (Poyarkov et al., 2018). The species was subsequently listed as Endangered in the IUCN Red List of Threatened Species due to habitat loss and degradation (IUCN, 2023).

As a result of our recent field surveys in Son La Province, individuals of *Micryletta* were collected and their morphological characteristics resemble the description of *Micryletta nigromaculata* by Poyarkov et al. (2018). We herein confirm to record the species for the first time from Son La Province. In addition, to better understand the natural history of dietary ecology of the highly threatened 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).

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

Locality	Date	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 Commune	16-19 June 2017	6	9	15
	15-18 July 2017	8	6	14
	11-13 September 2017	3	3	6
Total		27	25	52

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.

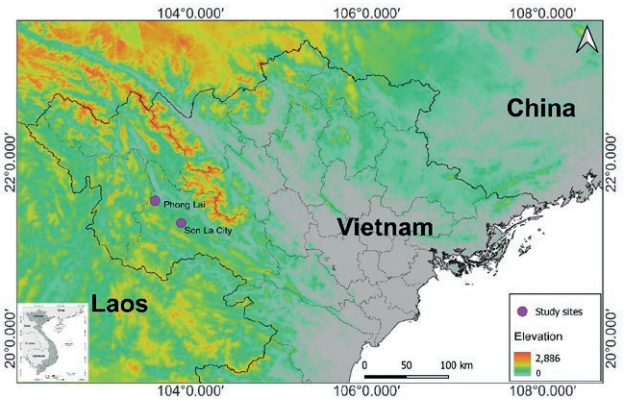

Fig. 1. Map of surveyed sites in Son La Province, northwestern Vietnam: (1) Son La City and (2) Phong Lai Commune.

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, mm^3) 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: $D = \Sigma[n_i(n_i - 1)]/[N(N - 1)]$; 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: $H' = -\Sigma(p_i \times \ln p_i)$; 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).

Shapiro-Wilk's test was used to check the assumption of normality. Wilcoxon tests were performed to determine differences or similarities in phenotypic characteristics of the species, and sizes and volume of ingested food between males and females, and between populations. Females were identified by having longer snout-vent length (SVL ≥ 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 nigromaculata *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. nigromaculata*. This is also the first record of *M. nigromaculata* in Son La Province, which is approximately 200 km from the type locality of the species in Cuc Phuong National Park of Ninh Binh Province.

Morphological characteristics of paddy frogs collected in Son La Province match well with the diagnosis of *Micryletta nigromaculata* (Poyarkov et al., 2018): size small (SVL: 14.8–27.7 mm, n = 52); head wider than long; snout obtusely rounded in profile; eyes equal to or shorter than snout; the interorbital distance wider than upper eyelid; tibiotarsal articulation of adpressed limb reaching the level of eye center; dorsal surface granular with small round tubercles; supratympanic fold present; outer metatarsal tubercle absent; dorsum coloration brown to reddish-brown; body flanks brown with dark-brown to black patches or spots edged with white, a large black blotch in the inguinal area on each side; lateral sides of head immaculate reddish brown lacking white patches; and throat in males whitish with light-gray marbling (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) and a narrower mouth width (MW: 5.6 ± 0.73 mm, ranging 4.4 – 6.7 mm, n = 27) 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; all P-values < 0.0001).

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 30ºC. Other sympatric amphibian species were also observed, including *Leptobrachella* 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. nigromaculata* 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).

Fig. 2. Natural habitat (above) and individuals (below) of *Micryletta nigromaculata* found in Son La Province, northwestern Vietnam.

In terms of size measurements, the item-mean width (W_m) of ingested prey was 1.33 ± 0.12 mm (0.2 – 5.0 mm), the item-total width (W_t) was 15.7 ± 2.56 (0.5 – 66.0 mm), the item-mean length (L_m) was 2.8 ± 0.19 mm (0.64 – 7.23 mm), and the item-total length (L_t) was 35.24 ± 6.6 (2.0 – 184.8 mm) (Fig. 3, Table 2). We calculated the mean volume of 44.02 ± 8.41 mm^3 (0.26 – 224.6 mm^3). There was only a significant difference in the W_m of consumed prey between males and females (P-value = 0.045), whereas remaining prey characteristics (i.e., W_t, L_m, L_t and volume) were relatively similar between intraspecific sexes and localities (all 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 phenotypic traits of SVL and MW of the species (Table 2).

Table 2. Sizes and volume of prey consumed by *Micryletta nigromaculata* in relation to phenotypic characteristics. Total = values computed on all individuals; Female = data for all females; Male = data for all males; Sex comparison = Wilcoxon's test for between-sex comparison; Locality comparison = Wilcoxon's test for between-localities comparison; SVL = Pearson's correlation between prey measures and snout-to-vent length; for all test, statistic and associated P-value are reported; significant values are bolded. Measures abbreviations: W_m = item-mean width; W_t = item-total width; L_m = item-mean length; L_t = item-total length, Volume = item volume.

	Total	Female	Male	Test (P-values)	Test (Locality)	SVL	HW
W_m (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)	**W = 166 P = 0.045**	W = 150.5 P = 0.34	r = -0.18 P = 0.25	r = 0.02 P = 0.92
W_t (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)	W = 237.5 P = 0.75	W = 229.5 P = 0.27	r = 0.14 P = 0.34	r = 0.05 P = 0.72
L_m (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)	W = 257.5 P = 0.90	W = 179 P = 0.84	r = 0.06 P = 0.69	r = 0.24 P = 0.11
L_t (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)	W = 262 P = 0.83	W = 254.5 P = 0.07	r = 0.23 P = 0.14	r = 0.14 P = 0.35
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 – 224.84)	W = 232 P = 0.66	W = 222 P = 0.36	r = -0.06 P = 0.70	r = -0.02 P = 0.91

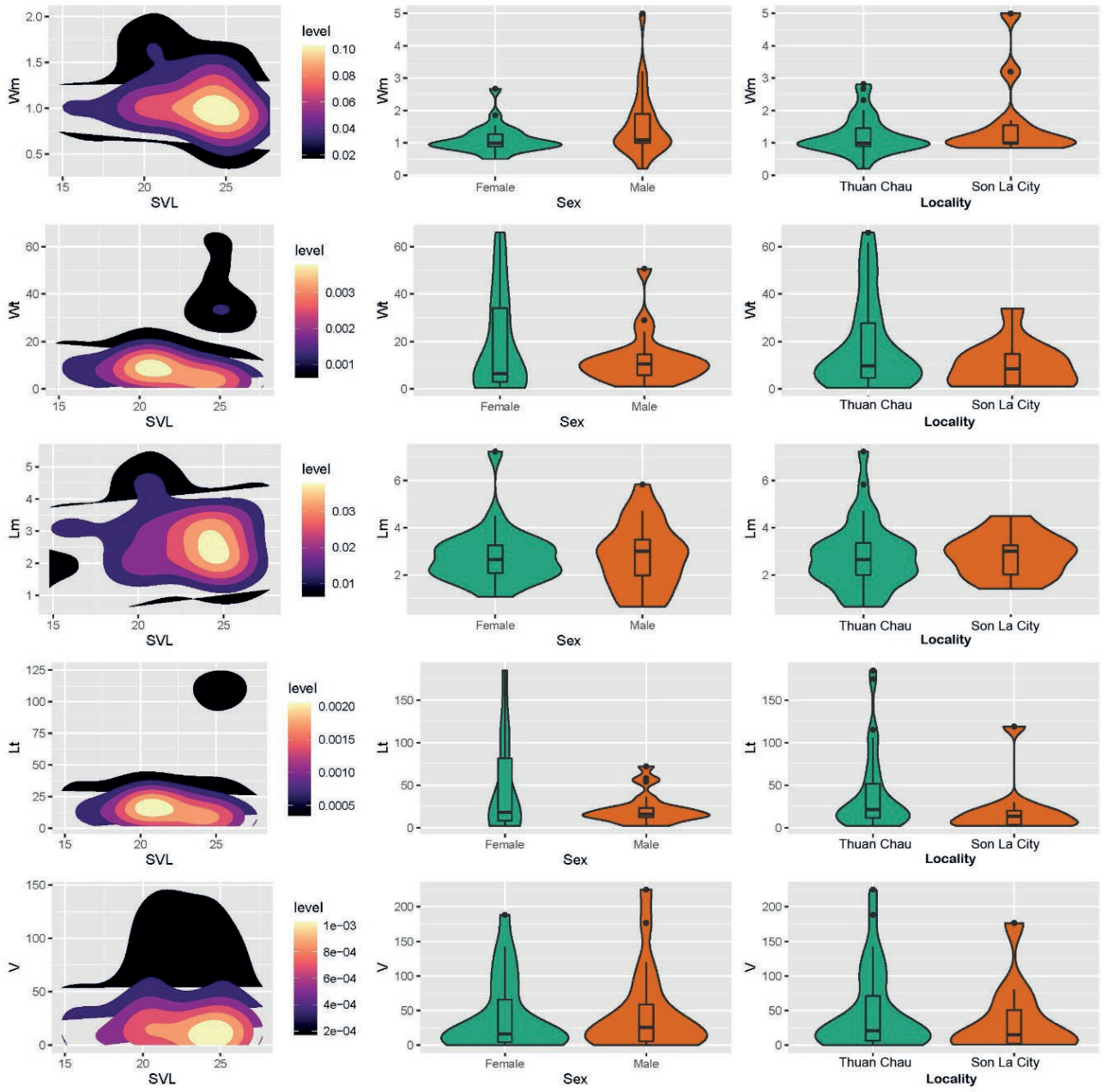

Fig. 3. Size and volume of prey consumed by *Micryletta nigromaculata* in relation to snout-vent length (left column), to sex (middle column), and locality (right column).

The prey of *M. nigromaculata* 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 more than 75% of Ix (Table 3).

The dietary breadth of *M. nigromaculata* 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. nigromaculata* had higher values of both Simpson's index (0.926) and evenness (0.783) than those of females (Table 4). The population of *M. nigromaculata* in Son La City consumed less diverse prey than the population in Thuan Chau District (Table 4).

Table 3. Dietary composition of *M. nigromaculata* in Son La Province: F = frequency of occurrence, N = number of items, V = volume (mm^3), 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. Diversity and Evenness indices of *Micryletta nigromaculata*'s prey, expressed with value (95% Confidence limits).

	Simpson 1-D	Evenness
Species (*Micryletta nigromaculata*)	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)

DISCUSSION

In this study, *M. nigromaculata* was first recorded in Son La Province, which is approximately 200 km and 350 km far distance from the type locality of the species in Ninh Binh Province and Hai Phong City, respectively. From Son La Province, the species has been recorded at significantly higher elevations up to 700 m a.s.l., whereas the species was previously known at elevations of 90 – 150 m a.s.l. only (Poyarkov et al., 2018). Our finding agrees well with the anticipation of Poyarkov et al. (2018) that this species can occur at different elevations in other adjacent ecosystems of limestone karst in northern Vietnam. With the first-time record of *M. nigromaculata* in Son La Province, we recommend additional field surveys should be conducted in other karst ecosystems in northern Vietnam to discover new populations of the species and fully understand the biogeographical aspect.

A total of 12 different prey types belonging to six invertebrate orders, larvae and spiders (Araneae) were identified as the food spectrum of *M. nigromaculata*, 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. nigromaculata*. 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. nigromaculata*. Only 17 individuals of *M. nigromaculata* 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. Wild populations of the species in Son La Province ergo have been severely threatened by habitat loss and limestone extraction (Pham pers. obs.). Such human impacts particularly destabilize its wild populations and food availability. The potential of finding new populations beyond the known areas of occupancy in northern Vietnam, as Poyarkov et

al. (2022) anticipated, could reduce the risk of extinction to this endangered species (IUCN, 2023). However, similar to what is happening in Son La Province, unsustainable human activities (particularly limestone extraction and tourist activities) over karst ecosystems in northern Vietnam, can extensively impact undiscovered populations of the range-restricted species in unique karstic habitats (Clemens et al., 2006; Poyarkov et al., 2022). Therefore, conservation measures would urgently be required, though *M. nigromaculata* 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 highly threatened species as well as its natural habitats. Besides the investigation of dietary ecology, we highly recommend further studies to identify the fully geographical extent, assess population status and determine other ecological traits of *M. nigromaculata*. Only when the background data becomes available, appropriate conservation measures can be designed to better protect the species from current extinction risks.

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