



## A description of the tadpole of *Megophrys* "*Brachytarsophrys*" *intermedia* (Smith, 1921)

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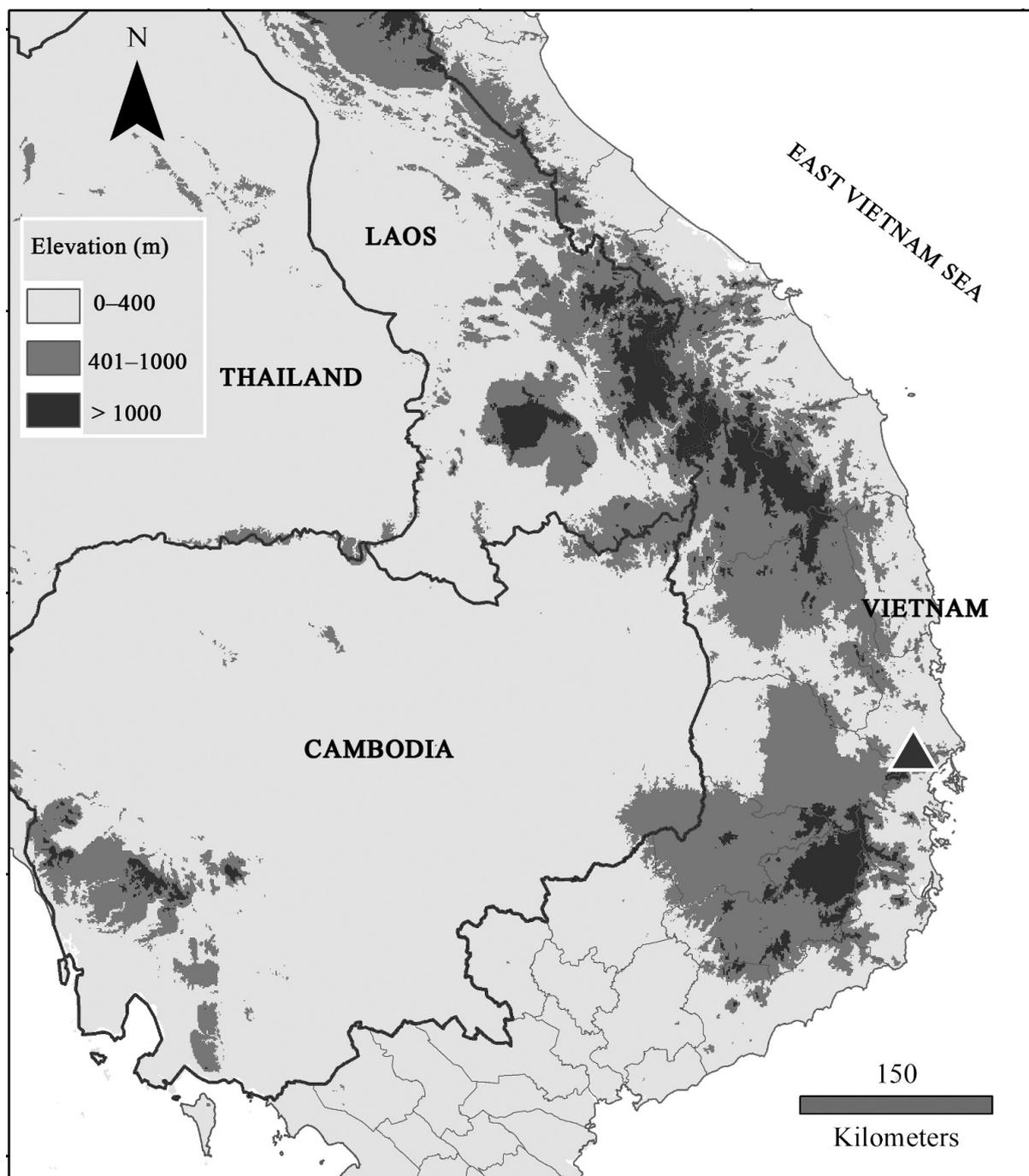
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*Megophrys* Kuhl and Van Hasselt are an Asian radiation of terrestrial frogs which are usually associated with montane forest (Mahony *et al.* 2017). The Annam horned frog (*Megophrys intermedia*) is reported from montane evergreen forest in Vietnam and Lao People's Democratic Republic (Stuart 2005; Hendrix *et al.* 2008; Nguyen *et al.* 2009; Tran 2013; Orlov *et al.* 2015; IUCN 2017; Mahony *et al.* 2017). The IUCN Red List assessment for the species reports an elevational range of 782–1500 m asl (IUCN 2017) although the type locality was reported to be above 1500 m asl (Smith 1921), and Tran (2013) collected a specimen at 1515 m asl. *Megophrys intermedia* is assessed as Least Concern (IUCN 2017) and little is known about the reproductive behaviour and life history of the species. In Lam Dong Province (Vietnam), male *M. intermedia* call in March (Tran 2013); on the Langbian Plateau (Vietnam), male specimens are reported to call by day and by night from March to May and specimens called from within deep crevices between boulders in streams (Smith 1921); in Xe Kong Province (Laos), males call or guard egg clutches that are laid in water in July (Stuart 2005).

We present a description of the tadpole of *M. intermedia* based on four specimens (ITBCZ 5913; ITBCZ 5912; ITBCZ 4590 and ITBCZ 5914) collected at night from a small, slow moving stream with a water temperature of 21.5 °C in evergreen secondary forest in Song Hinh Protected Forest, Song Hinh District, Phu Yen Province on the eastern slopes of the Langbian Plateau in southern Vietnam (12°47'02.4"N 109°02'13.2"E, 362 m asl; Figs. 1 and 2) on the 25<sup>th</sup> of March 2019 at 21:15. At the time of collection, approximately 15 tadpoles were active and feeding at the water surface and they were found in a still water stretch at the edge of a stream. The maximum depth of the stream was approximately 1 m, and the tadpoles were feeding in an area that was 0.2–0.4 m in depth (Fig. 2A). The substrate consisted of rocks embedded in sediment and a layer of submerged leaf litter (Fig. 2B). When the tadpoles were disturbed, they retreated under the submerged leaf litter. We did not attempt to find tadpoles during the day. Adult *M. intermedia* were not seen or heard at the collection site. Male frogs of the subgenus *Brachytarsophrys* call before and after heavy rain (e.g., Wogan *et al.* 2004), there had been no rain for several days preceding our visit to the site. There were no signs of human disturbance or pollution at the collection site. Specimens were photographed in life before being humanely euthanised using a 20% solution of benzocaine which was dissolved in stream water held in food grade plastic bags of water containing live tadpoles. Tissue samples (tail clips) for molecular analyses were extracted from freshly euthanised specimens and stored in absolute ethanol prior to fixation of specimens with 10% formalin for 24 hours and subsequent storage in absolute ethanol. Specimens were subsequently deposited at the Institute of Tropical Biology Zoological Collection (ITBCZ), Ho Chi Minh City, Vietnam.

Total DNA was extracted from the tail muscle of one tadpole using standard phenol-chloroform extraction (Hillis *et al.* 1996), followed by isopropanol precipitation. A 447-bp length fragment of 16S rRNA mitochondrial gene was amplified using ScreenMix-HS (Evrogen, Russia) following the manufacturer's instructions. The PCR contained 6 µL of ScreenMix-HS, 21 µL of water, 0.9 µL of each primer at a concentration of 10 pmol/µL, and 1.2 µL of template DNA at a concentration up to ca. 100 ng DNA/µL in a 30 µL reaction volume. The primers used for PCR and sequencing were 16sl2021 (5'-CCT ACC GAG CTT AGT AAT AGC TGG TT-3'; Tominaga *et al.* 2006 modified from Hedges 1994) and 16SH-1 (5'-CTC CGG TCT GAA CTC AGA TCA CGT AGG-3'; Hedges

1994). The PCR conditions followed Poyarkov *et al.* (2015). The obtained PCR products were sent to the Institute of Biotechnology (ITB, Hanoi, Vietnam) for subsequent purification (using MEGAquick-spin™ Plus Total Fragment DNA Purification Kit) and sequencing. The newly obtained sequence was initially verified by eye using DNA Baser v4.20.0, and the edited sequence was deposited in GenBank (Benson *et al.* 2017) under the accession number MT651607.



**FIGURE 1.** Map of southern and central Vietnam, Cambodia, northeast Thailand, and southern Laos. The black triangle represents the tadpole collection site in Phu Yen Province.

The new sequence was then checked on BLAST (NCBI) to verify approximate identity. Uncorrected  $p$ -distance (calculated in MEGA 7.0, Kumar *et al.* 2016) between the sequence in this study and a sequence from *M. intermedia* collected approximately 500 km to the northeast in Phong Nha Ke Bang National Park, Quang Binh Province (GenBank accession numbers; HQ588950.1) was 1.4%. Before calculating  $p$ -distance, both sequences were checked by eye for errors then aligned together using the ClustalW (Thompson *et al.* 1997) option in MEGA 7.0.



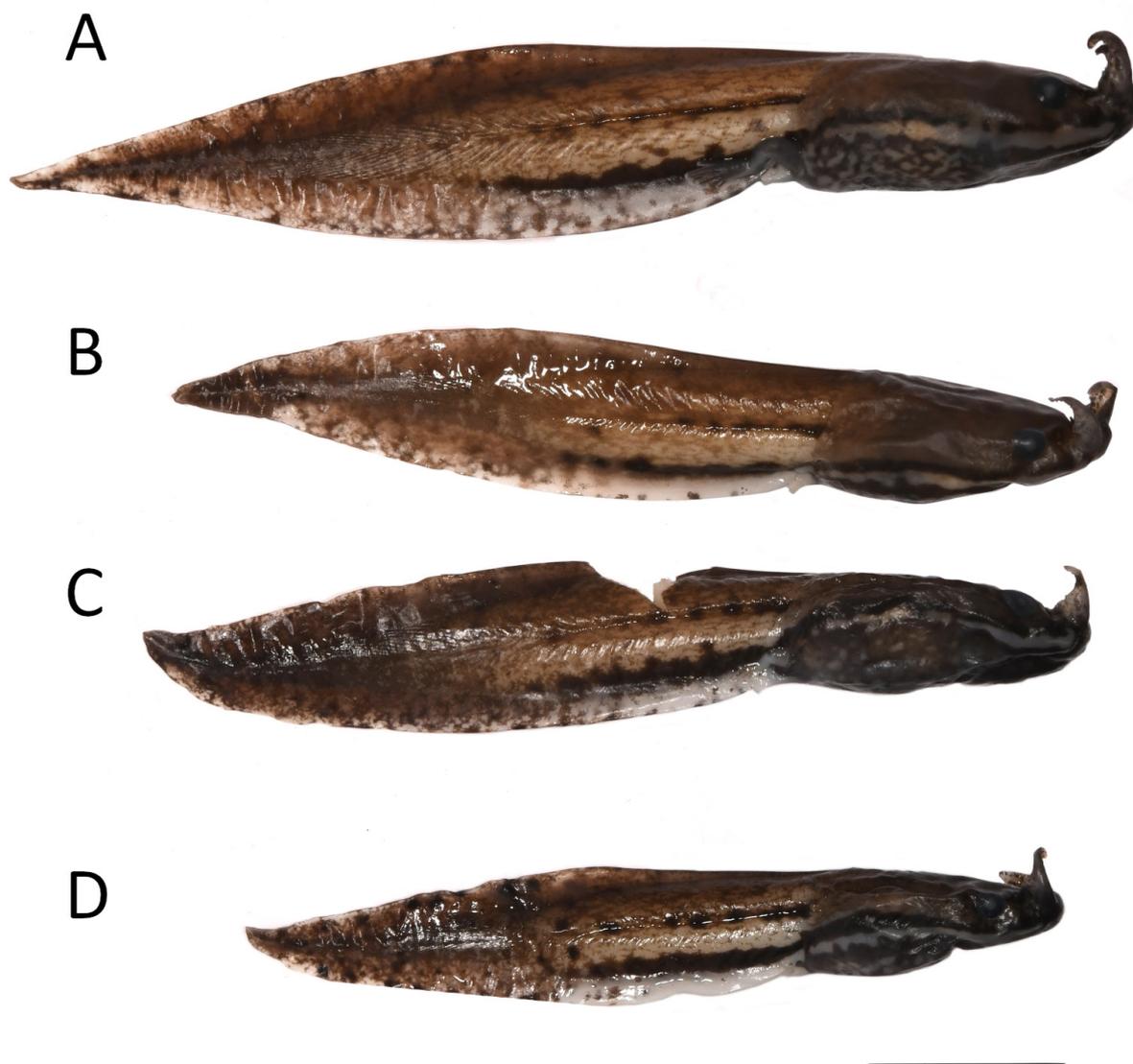
**FIGURE 2.** (A) Macrohabitat of *Megophrys intermedia* tadpoles in Song Hinh District, Phu Yen Province, Vietnam; (B) microhabitat of *Megophrys intermedia* tadpoles in Song Hinh District, Phu Yen Province, Vietnam.

We used ImageJ 1.49 (Schneider *et al.* 2012) to measure preserved tadpoles from photographs. Staging followed the Gosner (1960) table, and we used Altig and McDiarmid (1999) for the terminology for the tadpole description. Measurements and morphometric abbreviations follow Oberhammer *et al.* (2014) and are as follows: body length from snout to the point where the axis of the tail myotomes touches the body wall (BL); maximal body height (BH); body end to the centre of the spiracle (BS); maximal body width (BW); eye diameter (ED); eye to snout distance (ES); internarial distance measured from the centre of each nare (IND); interorbital distance measured from centre of each eye (IOD); lower fin height at the point of the maximal tail height (LFH); maximal tail height, including fins (MTH); distance from the centre of the nare to the centre of the eye (NE); oral disc width (ODW); distance from the centre of the nare to the snout (SN); distance from the snout to the centre of the spiracle (SS); total length (TTL); tail length = TTL–BL (TAL); maximal tail muscle height at the body–tail junction where the ventral line of the musculature meets the contour of the trunk (TMH); maximal tail muscle width (TMW); and upper fin height at the point of maximal tail height (UFH).

The body is depressed and longitudinally oval shaped (Figs. 3 and 4); BL/BW = 55.6% (52.0–60.5%,  $N=4$ ); the rims of the anterolaterally opening nares are raised from the body wall and are closer to the eyes than they are to the snout; the internarial distance is less than the interorbital distance; the eyes are positioned dorsolaterally, the pupils are round; the spiracle is sinistral, the spiracular tube protrudes from approximately two thirds of the way along the body wall, slightly anterior to the maximal trunk width, the spiracular tube opens laterally; the mean tail length is 69.5% (67.9–70.4%,  $N=4$ ) of the total length; the maximal tail height is approximate to the mid length of the tail; the dorsal tail fin inserts posteriorly to the body–tail junction, the dorsal fin is low, particularly along the proximal half of the tail length; the maximal tail muscle width is 52.0% (48.4–56.4%,  $N=4$ ) of the maximal width of the body, the tip of the tail is pointed; the oral disc is subterminal and opens antero-dorsally, the lateral corners of the oral disc fold medially and turn upward when they are not fully extended (and in preservative), the mean width of the umbelliform oral disc makes up 78.0% (68.1–89.2%,  $N=4$ ) of the maximal width of the body; in life, the maximal body width is 50.7% the maximal width of the fully extended oral disc width ( $N=1$ ; Fig. 5); the lower lip is bi-triangular shaped when mouthparts are fully expanded in life, marginal papillae are absent, and there are four rows of submarginal papillae on both the upper and lower lip when they are counted medially at the maximal width of the oral disc; these are regularly positioned, longitudinally oval shaped on the three inner rows and they become smaller and circular in shape on the outermost row, medially the lower lip is deeper than the upper lip; labial teeth are absent; the upper jaw sheath is weakly serrated with medial notch, the lower jaw sheath has a medial v-shaped protrusion which is directly opposite to the medial notch on the upper jaw sheath. Table 1 summarises tadpole measurements.

In life, the body is light brown with a darker brown medial saddle; the neuromasts are pale brown; the area surrounding the nares is dark brown, the darker pigmentation forming a blotch that extends to the outer surface of the closest eye; the oral disc is a translucent tan brown with dark brown submarginal papillae; the tail is light brown, at the point where the tail meets the body there is a very dark brown irregular stripe that extends from the body–tail junction and this stripe runs along the midline of the lateral tail surface, this stripe fades to light brown half way

along the length of the tail; the lower margin of the tail has an irregular shaped black line which extends from the body to one third the length of the tail; the upper tail fin is light brown at the point it meets the body and becomes opaque and speckled with dark brown half way along the tail length, this speckling continues to the tip; the lower fin is opaque at the point that it meets the body and is speckled with dark brown half way along the tail length and this speckling continues to the tip; the ventral and lateral sides of the body are a bluish- grey with blue iridescence, the bluish-grey ventral surface of the body are speckled with white; the ventrolateral surface of the head and body has a longitudinal clean white stripe bordered with black; this starts beneath the oral disc and extends along the body to the spiracle, after the spiracle this white stripe is broken into several white blotches surrounded by black; the vent tube is white; the gills and coils of the digestive tract are not visible through the ventral skin; the sclera of the eye is black with green-gold flecks; the pupil is black and rounded; the iris is green-gold and speckled with black dots (see Figs. 4 and 5).



**FIGURE 3.** Lateral view of *Megophrys intermedia* tadpoles in preservative: (A) ITBCZ 5913; (B) ITBCZ 5912; (C) ITBCZ 4590 and (D) ITBCZ 5914. 10 mm scale.

In preservative the oral disc is translucent grey brown with darker brown submarginal papillae; the body is dark brown, the tail is lighter brown becoming darker towards the tip, the white line and blotches on the lateral and ventral surfaces appear grey in preservative; the venter is grey; the iris is dark grey (Fig. 3).

**TABLE 1.** Measurements (in mm) of *Megophrys (Brachytarsophrys) intermedia* tadpole specimens.

Specimen	ITBCZ 5913 (SH3)	ITBCZ 5912 (SH2)	ITBCZ 4590 (SH1)	ITBCZ 5914 (SH4)
GenBank accession no.	N/A	N/A	MT651607	N/A
Gosner Stage	39	32	36	32
BL	16.3	15.5	15.0	12.4
BH	7.0	6.8	6.1	5.1
BS	6.8	6.8	6.6	6.0
BW	9.1	8.4	7.8	7.5
ED	1.9	1.8	1.7	1.5
ES	3.5	3.3	3.0	2.6
IND	3.8	3.2	3.4	3.5
IOD	6.0	5.4	5.4	5.2
NE	1.7	1.0	1.0	0.7
SN	2.3	2.6	1.8	2.1
SS	10.2	8.1	8.7	6.7
ODW	6.2	5.7	5.4	6.4
LFH	2.1	2.5	2.1	1.9
MTH	9.7	8.4	7.8	7.1
TAL	38.8	32.8	33.7	29.3
TTL	55.1	48.3	48.7	41.7
TMH	5.3	5.2	4.3	4.1
TMW	4.4	4.3	4.4	3.9
UFH	2.4	2.4	1.8	1.7
ODW in life	Not recorded	Not recorded	15.4	Not recorded

**FIGURE 4.** *Megophrys intermedia* tadpole ITBCZ 4590 in life: (A) lateral view; (B) dorsal view; (C) lateroventral view. 10 mm scale.

**TABLE 2.** Measurements of *Megophrys intermedia* (subgenus *Brachytrypophrys*; values in mm) tadpole specimens. \*Indicates that only mean values were provided in the referred publication.

Species	Gosner Stage	N	BL	TTL	TAL	BH:BW	BL:TAL	ODW:BW	Transverse stripe across ventral surface of abdomen	Reference
<i>M. chuannanensis</i>	26–27	Not reported	11	33.0	22.0	Not reported	Not reported	Not reported	Present	Fei & Ye 2001; Fei <i>et al.</i> 2012
<i>M. feae</i>	44	1	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	Several	Li <i>et al.</i> 2020
<i>M. intermedia</i>	32	2	12.4–15.5	41.7–48.3	29.3–32.8	68.0–81.0	42.3–47.3	67.9–85.3	Absent	Zhao <i>et al.</i> 2014
	36	1	15.0	48.7	33.7	78.2	44.5	69.2	Absent	This study
	39	1	16.3	55.1	38.8	76.9	42	68.1	Absent	This study
<i>M. orientalis</i>	36	1	12.3	Not reported	27.6	Not reported	44.6	Not reported	Absent	Li <i>et al.</i> 2020
<i>M. platyparietus</i>	38–39	10	Not reported	49.9*	Not reported	79.1*	47.8*	58.8*	Present	Li <i>et al.</i> 2011
<i>M. popei</i>	26	9	8.1 (7.0–9.1)	Not reported	20.2 (15.7–21.9)	Not reported	40.1*	Not reported	Present	Zhao <i>et al.</i> 2014
	27	3	8.1 (7.5–9.4)	Not reported	19.1 (17.2–21.9)	Not reported	42.4*	Not reported	Present	Zhao <i>et al.</i> 2014
	29	2	10.2 (10.1–10.3)	Not reported	20.8 (19.9–21.7)	Not reported	49.0*	Not reported	Present	Zhao <i>et al.</i> 2014

We compare the tadpole of *M. intermedia* with the tadpoles of five of the six species within the subgenus *Brachytarsophrys* Tian and Hu: *M. chuannanensis* (Fei, Ye & Huang); *M. feae* (Boulenger); *M. orientalis* (Li, Lyu, Wang & Wang); *M. platyparietus* (Rao & Yang) and *M. popei* (Zhao, Yang, Chen, Chen & Wang). The tadpole of *M. carinense* (Boulenger) has not been described and no comparison can be made. The tadpole of *M. intermedia* at Gosner Stages 32–39 differs from the tadpole of *M. chuannanensis* (Gosner Stages 26–27), *M. platyparietus* (Gosner Stages 38–39) and *M. popei* (Gosner Stages 26–29) by the absence of a transverse white stripe across ventral surface of abdomen, versus presence in *M. chuannanensis* (Fei & Ye 2001), *M. platyparietus* (Li *et al.* 2011) and *M. popei* (Zhao *et al.* 2014). The tadpole of *M. intermedia* at Gosner Stages 32–39 differs from *M. feae* (Gosner Stage 40) by the absence of a transverse white stripe across ventral surface of abdomen (versus several white stripes across ventral surface of abdomen in *M. feae*; Zhao *et al.* 2014). There are no obvious differences between the tadpole of *M. intermedia* and *M. orientalis*. A summary of the morphological differences between the tadpoles of *M. intermedia* and the tadpoles of other species in the subgenus *Brachytarsophrys* is presented in Table 2.



**FIGURE 5.** Oral disc of *Megophrys intermedia* tadpole ITBCZ 4590 in life. 3.5 mm scale.

The clean white longitudinal stripe bordered with black on the ventrolateral surface of the head and body is present in *M. intermedia* (Figs. 3 and 4A), *M. chuannanensis*, *M. orientalis*, and *M. popei* (Li *et al.* 2020), but not mentioned in the very brief tadpole description of *M. feae* (Fei *et al.* 2012). This pattern has not been reported in any of the other *Megophrys* subgenera: *Atympnanophrys* Tian and Hu (e.g., Li *et al.* 2011); *Ophryophryne* Boulenger (e.g., Poyarkov *et al.* 2017); *Panophrys* Rao and Yang (e.g., Wang *et al.* 2014; Tapley *et al.* 2017; Li *et al.* 2019; Tapley *et al.* In Press) *Pelobatrachus* Beddard (e.g., Malkmus *et al.* 2002); *Xenophrys* Günther (e.g., Stuart *et al.* 2006; Deuti *et al.* 2017; Tapley *et al.* In Press) or in *Megophrys dringi* Inger, Stuebing and Tan, which has not been assigned to a subgenus (Oberhammer *et al.* 2014). There are no published descriptions of tadpoles from the subgenus *Megophrys* Kuhl and Van Hasselt and so we tentatively suggest that the presence of this white longitudinal stripe could be considered diagnostic of the subgenus *Brachytarsophrys*.

The site where the tadpoles were collected in this study is the lowest published elevation record for the species; 640 m lower than the lowest elevation reported in the IUCN Red List assessment (IUCN 2017) and 420 m lower than reported by Tran (2013). The Current Extent of Occurrence of *M. intermedia* is estimated to be 156752 km<sup>2</sup> (IUCN 2017) but this is likely to be an underestimate given that the species occurs at much lower elevations than previously reported. There are published reports of male *M. intermedia* calling in March, May and July in Vietnam

and Laos respectively (Smith 1921; Stuart 2005; Tran 2013). The presence of relatively late stage tadpoles in March indicates that the species may have a more prolonged and earlier breeding season than currently known or it could also suggest that the tadpoles have extended larval periods.

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