



## A description of the tadpole of the Critically Endangered Botsford's leaf-litter frog (*Leptobranchella botsfordi*) with comments on the distribution and conservation status of the species

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We describe the tadpole of the Botsford's leaf-litter frog *Leptobranchella botsfordi*, a species endemic to Mount Fansipan in northwest Vietnam, for the first time. Tadpoles of this species were found in steep, fast-flowing streams at elevations between 2500–2600 m asl. We also report a previously unknown locality for this species on Mount Fansipan, which increases the species' known Extent of Occurrence from 8 km<sup>2</sup> to 36 km<sup>2</sup>. Our findings will inform subsequent conservation initiatives for this poorly known and highly threatened species.

*Leptobranchella botsfordi* (Rowley, Dau, & Nguyen) was described in 2013; at the time of description it was known only from its type locality (Hoang Lien National Park, Mount Fansipan, Sa Pa District, Lao Cai Province, Vietnam; between 2795–2815 m asl.). The species' Extent of Occurrence (EOO; IUCN SSC 2012) is estimated to be 8 km<sup>2</sup> (IUCN SSC 2015a; Rowley *et al.* 2013). The habitat at the type locality faces ongoing degradation and pollution related to the activities of tourism and associated infrastructure development and gravel mining (Rowley *et al.* 2013; Tapley *et al.* 2017). As a result, the species is assessed as Critically Endangered by the IUCN (IUCN SSC 2015a). *Leptobranchella botsfordi* is molecularly divergent from other species in the genus (Chen *et al.* 2018) and is included in the top 100 Evolutionarily Distinct and Globally Endangered amphibians (Gumbs *et al.* 2018). The species is therefore considered a global priority for conservation. We describe the tadpole of this species based on two specimens collected at the type locality and a nearby locality; the latter represents an important range extension for this mountain-top endemic species.

Fieldwork was conducted from 2017–2019 at two sites on Mount Fansipan, Hoang Lien National Park, Sa Pa District, Lao Cai Province. Site 1, the type locality of the species (22.31482°N, 103.76867°E, 2617 m asl.; all coordinates hereafter recorded in datum WGS 84) was surveyed from 27–31 December 2017 and site 2, a stream on the Cat Cat trail 3.5 km away from site 1, (22.29474°N, 103.80297°E, 2578 m asl) was surveyed from 8–15 September 2018 and from 21–24 June 2019. We also surveyed two additional sites more than 20 km to the north of Mount Fansipan in Bat Xat Nature Reserve, Bat Xat District, Lao Cai Province; Mount Ky Quan San (22.49960°N, 103.60170°E, 2685 m asl.) on 09 September 2017 and the north-eastern slopes of Mount Pu Ta Leng (22.42620°N, 103.62120°E, 2345 m asl.) on 07 March 2018 and 04 October 2019. Tadpoles were collected at night using a handheld net and photographed in life (with a scale) before being humanely euthanised using a 20% solution of benzocaine (Torreilles *et al.* 2009) which was added to bags of stream water containing tadpoles. Tissue samples (tail clips) for molecular analyses were extracted from freshly euthanised specimens and stored in EDTA/DMSO prior to fixation and storage of specimens in 10% formalin. Specimens were deposited at the Vietnam National Museum of Nature, Hanoi (VNMN).

We used ImageJ 1.49 (Schneider *et al.* 2012) to measure the tadpoles from the photographs (in life and preservative). Staging followed Gosner (1960), and tadpole terminology is that of Altig & McDiarmid (1999). Detailed morphometric measurements are shown in Table 1. The oral disc of the tadpole was drawn under microscope with scale (Tiger TG-390M 3X–90X, China) then edited using Adobe Illustrator CC 2018 software (San Jose, California, USA).

Four species of the genus *Leptobranchella* have been reported from Mount Fansipan; *L. botsfordi*; *L. bourreti* (Dubois); *L. ventripunctata* (Fei, Ye & Li) and *L. phuvialis* (Ohler, Marquis, Swan & Grosjean) and the latter three species are known to be sympatric (Ohler *et al.* 2000, 2011; Chen *et al.* 2018; our data). Of these three species, only the tadpole of *L. bourreti* has been described (Ohler *et al.* 2011), and we compare the tadpole of *L. bourreti* to the tadpole of *L. botsfordi* described herein.

A species distribution map was created in ArcMap 10.2.2 using the ESRI World Topographic basemap layer, WorldClim altitude raster (Hijmans *et al.* 2005) and IUCN Elevation raster to visualise topography, and the ESRI World Imagery basemap layer to determine local land cover. The elevation range was estimated by adding a 50 m buffer to the lowest and highest known elevation records of the species following the standardised procedure developed by the mainland Southeast Asia Regional Red List Authority. Extent of Occurrence (EOO) was measured using the IUCN EOO Calculator tool v1.2 (IUCN SSC 2012).

Salifert test kits (Salifert, Duiven, The Netherlands) were used to measure nitrite, nitrate, alkalinity and the phosphate content of the water. Samples were taken in the mid-section of the stream at a depth of 10 cm and tests were carried out following the manufacturer's guidelines.

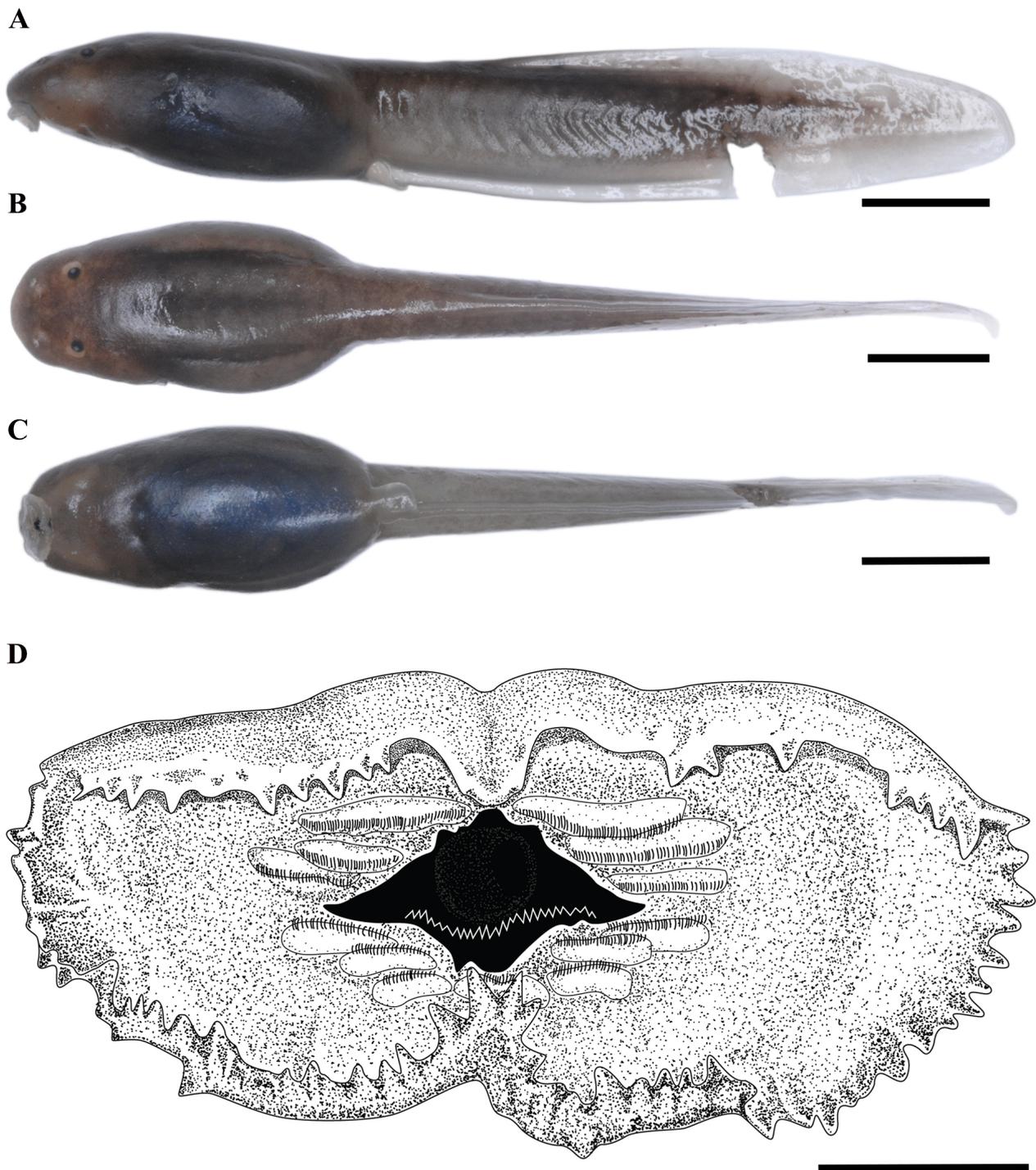
Total genomic DNA was extracted from the newly collected tissue samples with DNeasy tissue extraction kits (Qiagen), and we used the primers 16SAR and 16SBR of (Palumbi *et al.* 1991) to amplify 552 base pairs of the 16S rRNA gene. Standard PCR protocols were used, and PCR products were purified with ExoSap-IT (USB Corporation, OH, USA). Purified templates were sequenced directly by Macrogen (Seoul, Korea). Sequences were validated with Sequencher 4.10 (Gene Codes, Ann Arbor, MI), aligned with the ClustalW option in MEGA 7 (Kumar *et al.* 2016), and refined by eye. The new sequences were then checked on BLAST (NCBI) to verify their approximate identity. The newly obtained sequences were deposited in GenBank under accession numbers MT896136 and MT896137.

*Species confirmation:* Sequences generated from the two tadpoles were identical to those from the adult *L. botsfordi* holotype collected at Site 1 in 2012 (VNMN 03682, GenBank accession number MH055953). A single metamorph was also observed at site 2 and the morphological characters of this individual were congruent with adult *L. botsfordi* (Rowley *et al.* 2013).

*Morphology:* The description of tadpole morphology is based on two specimens (Figs. 1 and 2, Table 1.): VNMN 010898 (collected at Site 1, GenBank accession numbers MT896136), at Stage 25; and VNMN 010899 (collected at Site 2, GenBank accession numbers MT896137) at Stage 26. Body elongated (BW 46–49% BL), with rounded snout (Fig. 1A–C); nares anterodorsally positioned, nares closer to the tip of snout than to eyes; eyes positioned dorsolaterally, eyes comparatively small but distinct (ED 3–4% of BL); body laterally depressed (BH 71–79% of BW); conical tube shaped sinistral spiracle, spiracle positioned anteriorly to the widest part of the body, conical tube of spiracle is fused to body with a free short distal portion; tail muscle long, tail length approximately twice the body length (TL 176–200% of BL), rounded tail tip, tail musculature distinct, running parallel from tail base to anterior two thirds of tail length, before gradually tapering towards tail tip, tail fins relatively low, both upper and lower tail fins reach maximum height two thirds of the way along the tail length; cup-like oral disc, anteroventrally positioned (ODW 36–37% of BW), oral disc fringed with short pointed conical papillae; labial tooth row formula: 3(1–3)/4(1–3), jaw sheaths black, robust; upper jaw and lower jaw sheaths developed with distinctly serrated edges (Fig. 1D). An unvouchered specimen (observed at Site 2 on 23 June 2019) at Gosner Stage 44 had a total length 32.4 mm, four limbs fully developed, snout rounded, tympanum rounded, dorsum smooth with numerous tubercles, ventral surfaces smooth. Thickened tail 15.1 mm in length.

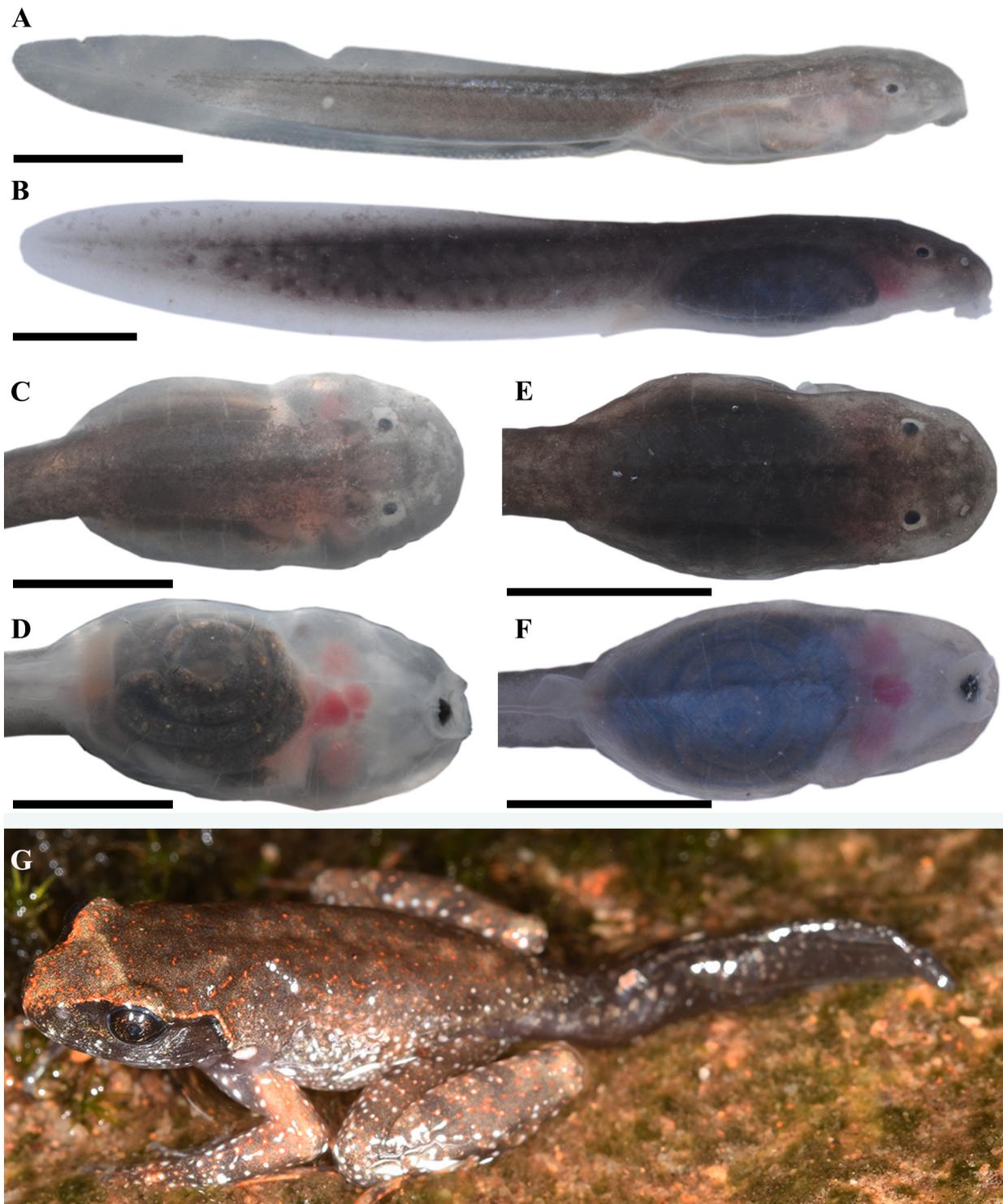
*Colour in life:* Tadpoles with whitish-brown to grey body with obvious, whitish neuromasts arranged in lines concentrated around nares and eyes, two lines of neuromasts present, running dorsolaterally along the body before continuing along lateral surfaces of tail muscle; iris black; internal gills distinctly reddish; tail muscle dark grey to whitish brown, tail fin whitish-brown; translucent skin on ventral surface of body, gut coil clearly visible (Fig. 2). The colour of VNMN 010898 is lighter than VNMN 010899 (Fig. 2). The unvouchered metamorph at Stage 44 had a brown dorsum with numerous reddish-orange spots on tubercles, border of tympanum reddish-orange, dorsolaterally light brown with white spots, ventral surfaces light brown with whitish spots, macroglands on body (including supra-axillary, pectoral, femoral and ventrolateral glands) whitish colour (Fig. 2G).

Colour of tadpoles in preservative: body and tail musculature blackish brown; tail fin similar to colouration in life (Fig. 1). The variation of morphological characters in the two tadpoles is shown in Table 1.



**FIGURE 1.** Tadpole of *Leptobrachella botsfordi* in preservative (VNMN 010899, Stage 26): A–C, lateral, dorsal, and ventral views; and D, oral disc. Scale bar is 0.5 cm in A–C and 0.5 mm in D.

*Comparison:* The tadpole of *L. botsfordi* at Gosner Stage 25–26 differs from *L. bourreti* at Gosner Stage 25–31 by having shorter length (TTL = 28.3–39.7 mm,  $N=2$  vs. 38.2–59.2 mm,  $N=11$  in *L. bourreti*; Ohler *et al.* 2011); smaller eyes (ED/BL 3.3–4.2%,  $N=2$  vs. 6.3–10.1%,  $N=10$  in *L. bourreti*; Ohler *et al.* 2011), shorter upper fin (UFH/MTH 19.3–26.7%,  $N=2$  vs. 29–35%,  $N=10$  in *L. bourreti*; Ohler *et al.* 2011), and colour in life (body whitish-brown to grey without spots on dorsal surface of head vs. body olive grey with lighter spots on dorsal surface of head in *L. bourreti*; Ohler *et al.* 2011).



**FIGURE 2.** Tadpoles of *Leptobrachella botsfordi* in life. A and B, lateral views of VNMN 010898 and VNMN 010899 (Stage 25 and 26 respectively); C and D, body dorsal and ventral view of VNMN 010898; E and F, body dorsal and ventral view of VNMN 010899; G, unvouchered specimen at Stage 44 (total length 32.4 mm) in life. Scale bar in A–F is 0.5 cm.

*Natural history:* Two *L. botsfordi* tadpoles were observed in total, one from Site 1 and the other from Site 2. These were both observed at night beneath rocks in small pools at the base of small waterfalls (these pools were about 1–2 m in width and 0.3–1.0 m in depth). The stream at Site 1 was on a steep gradient with a gravel and rock substrate, the stream width was 5–8 m at the point where the tadpole was collected. The riparian habitat was mixed bamboo forest and disturbed secondary broadleaf forest with a relatively open canopy. The stream at Site 2 was rocky and was also on a steep gradient; riparian vegetation consisted of mixed pine forest and evergreen forest and the stream width was about 3–4 m

at the point where the tadpole was collected. At Site 1, the pH was 6.0 and the GH was 30, and  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$ , and alkalinity all measured at 0.0 ppm. The water temperature was 9.2 °C (December 2017); at Site 2, only the air temperature was recorded (13.0°C, September 2018). The metamorph was observed at night on 23 June 2019 on a rock on the bank of the stream at Site 2. *Leptobranchella botsfordi* is thought to breed in June, males were calling in June in 2012, 2018 and 2019 (Rowley *et al.* 2013; authors, pers. obs.).

**TABLE 1.** Measurements of *Leptobranchella botsfordi* tadpoles and single metamorphosing specimen. All measurements are in millimetres. “–” missing data.

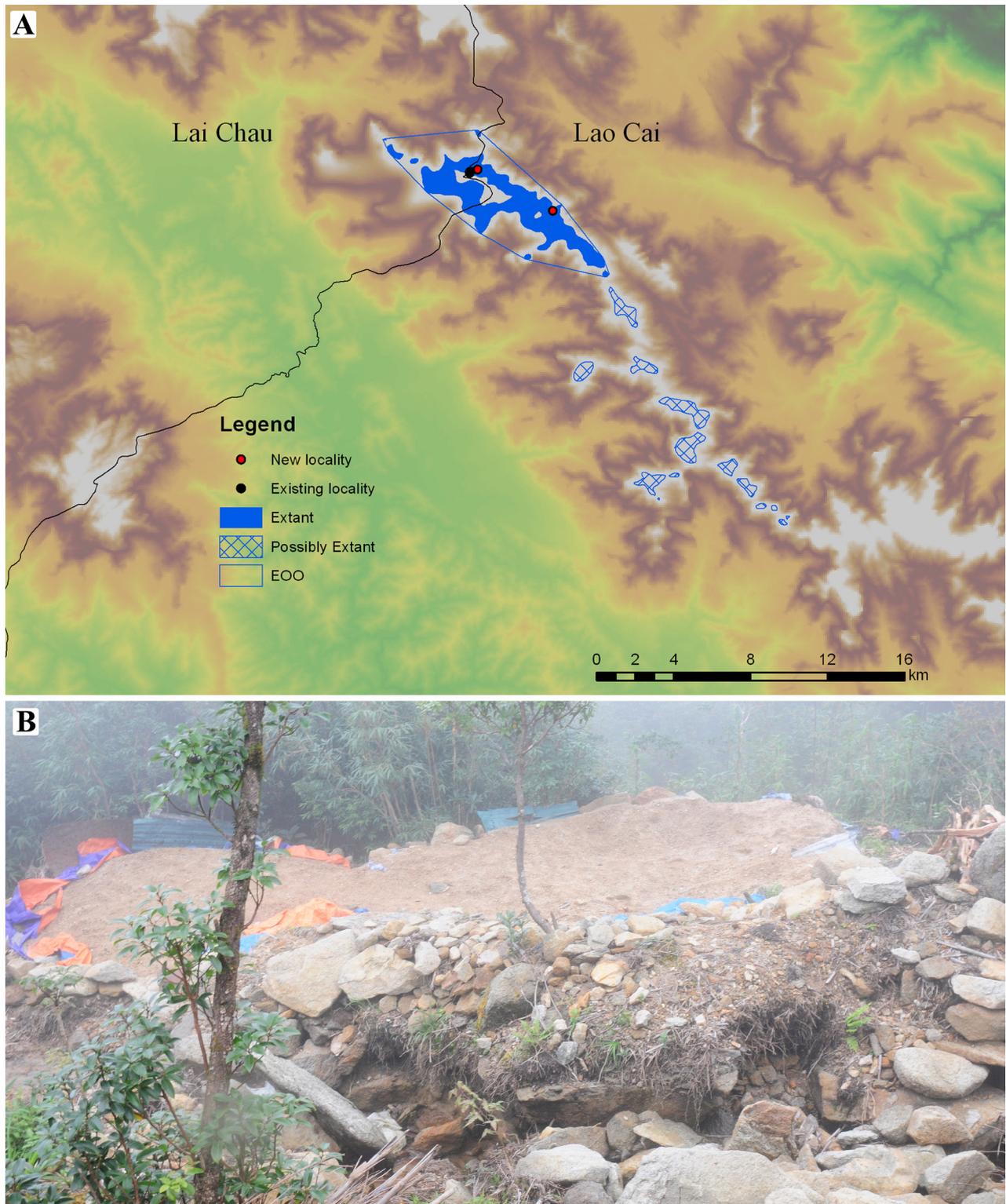
Specimen	VNMN 010898	VNMN 010899	Unvouchered metamorphosing individual
Gosner stage	25	26	44
Total length (TTL)	28.3	39.7	32.4
Body length (BL)	9.2	14.4	17.3
Maximal body height at trunk (BH)	3.0	5.5	–
Body end to centre of spiracle (BS)	3.4	8.6	–
Maximal body width (BW)	4.2	7.0	–
Eye diameter (ED)	0.3	0.6	1.6
Eye to snout distance (ES)	2.0	3.1	1.8
Internarial distance (IND)	1.5	2.5	2.1
Interorbital distance (IOD)	1.9	3.1	2.9
Lower fin height (LFH)	0.8	0.8	–
Maximal tail height, including fins (MTH)	3.0	5.7	–
Distance from centre of nare to centre of eye (NE)	1.0	1.8	1.8
Oral disc width (ODW)	1.5	2.6	–
Distance from centre of the nare to the snout (SN)	1.0	1.7	–
Distance from snout to centre of spiracle (SS)	3.5	6.5	–
Tail length = TTL–BL (TAL)	19.1	25.3	15.1
Maximal tail muscle height (TMH)	2.0	3.9	–
Maximal tail muscle width (TMW)	2.2	4.4	–
Upper fin height (UFH)	0.8	1.1	–

*Distribution and conservation status:* *Leptobranchella botsfordi* is only known from Mount Fansipan. Our new record is 3.5 km away from the type locality and at lower elevation than previously reported, expanding the known Extent of Occurrence for the species from 8 km<sup>2</sup> to 36 km<sup>2</sup> (Fig. 3A). The species is now known to occur between 2500–2815 m asl.

The description of the tadpole of *L. botsfordi* provides important new information on this Critically Endangered species and the habitat associations of the larvae. The adults appear seasonally active as we have only observed them from April to September and heard calling males from June to September. Tadpoles have been observed in September and December when temperatures were low and streams were not flowing as much as in warmer months, and water was only present in pools. This indicates that at least some tadpoles may overwinter and be present in streams throughout most of the year, increasing the utility of tadpole surveys in understanding the distribution and habitat requirements of this threatened species. Because our sample size was limited (N=2), additional specimens are needed for a more robust comparison with the tadpoles of other congeneric species on Mount Fansipan.

*Leptobranchella botsfordi* remains threatened by habitat degradation associated with tourism (IUCN SSC 2015a; Tapley *et al.* 2017). Indeed, we observed evidence that gravel mining is causing dramatic modification of the type locality of the species, likely to removing oviposition sites and impacting tadpole survival. The rate of gravel removal increased over our study period and this may be a particular issue for *L. botsfordi*, as stream-dwelling tadpoles typically have specific microhabitat requirements (Inger *et al.* 1986) and the tadpoles of many congeners are aquatic and fossorial and associated with gravel beds (Ohler *et al.* 2011). At Site 1, the single tadpole was found several hundred meters away from the area of the stream where the gravel is mined (Fig. 3B). No tadpoles were found at sites where gravel has been removed

(2795–2815 m asl.), despite intensive tadpole surveys. Alteration of the stream substrate and profile could therefore have a negative impact on tadpole development and population recruitment in this species. Removal of gravel may also temporarily suspend large amounts of sediment, resulting in reduced dissolved oxygen levels and siltation downstream, potentially affecting tadpoles over a wider area. We recommend that the gravel needed for construction and lining of trekking paths should not be sourced from streams that are important breeding sites for *L. botsfordi* and other threatened amphibian species.



**FIGURE 3.** A, The updated distribution range of *Leptobranchella botsfordi* in the Hoang Lien Range, northwest Vietnam. Solid blue area represents presumed range and cross-hatched blue area denotes areas where this species may be possibly extant. Blue outline denotes species' Extent of Occurrence. B, gravel mining at the type locality of *L. botsfordi* in September 2017.

Our discovery of a new locality for the species expands the Extent Of Occurrence for the species but *L. botsfordi* likely still qualifies as being listed as Critically Endangered (IUCN 2015a) in accordance with the IUCN Red List of Threatened Species categories and criteria B1ab (i,iii). The data herein are presented for easy assimilation into an updated Red List assessment for the species by the regional IUCN amphibian Red List authority. The discovery of a new site for the species is also significant as the site is at a stream not connected to the type locality (Site 1). Given the ongoing degradation at the type locality, we recommend that the new site be strictly protected and that and tourism activities are carefully monitored and managed.

The type locality of *L. botsfordi* is important breeding habitat for this species, but also for other range-restricted amphibians including the Critically Endangered *Oreolalax sterlingae*, Nguyen, Phung, Ziegler & Böhme 2013 (IUCN SSC 2015b; Rowley *et al.* 2017) and the newly described *Megophrys fansipanensis* Tapley, Cutajar, Mahony, Nguyen, Dau, Luong, Le, Nguyen, Nguyen, Portway, Luong & Rowley, which is likely to satisfy the criteria for being listed as Endangered (Tapley *et al.* 2018). The ongoing collection of sand and gravel for lining trekking routes and pollution related to the activities of tourists climbing Mount Fansipan is likely to be detrimental to this imperilled amphibian species assemblage.

We did not encounter *L. botsfordi* at Pu Ta Leng and Ky Quan San Mountains, Bat Xat Nature Reserve, Bat Xat District, Lao Cai Province, but these surveys were short in duration and more surveys are necessary in these areas in order to confirm the presence or absence of this species in these mountains. The true distribution, reproductive behaviour, and ecology of the species remain poorly known, and further research is recommended in order to inform future conservation decision making.

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