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Notes on oviposition sites and a significant range extension for Botsford's Leaf-litter Frog (*Leptobrachella botsfordi*)

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Leptobrachella botsfordi (Rowley et al., 2013) was described and scientifically named in 2013 from a single stream on Mount Fansipan, Hoang Lien National Park, Lao Cai Province, northwest Vietnam. It was later reported from an additional site approximately 4 km away on the same mountain. *Leptobrachella botsfordi* is only known to occur between 2578–2815 m elevation and currently, the Extent of Occurrence (IUCN SSC 2012) for the species is estimated to be just 36 km² (Nguyen et al., 2020). However, the range of this species remains poorly known and surveys to determine the true distribution of the species are a priority. Surveys in apparently suitable habitat to the northeast of the known range of the species, on Mount Pu Ta Leng and Mount Ky Quan San (20 km and 29 km to the northeast of Mount Fansipan respectively), failed to detect the species (Nguyen et al., 2021). However, potential habitat for the species exists to the south of known localities (Nguyen et al., 2020).

Leptobrachella botsfordi is listed as Critically Endangered (IUCN SSC, 2021a). The species is threatened by ongoing habitat degradation and pollution, most of which is the result of infrastructural developments associated with tourism (Rowley et al., 2013; Tapley et al., 2017; Nguyen et al., 2020; IUCN SSC, 2021a). Gravel mining is suspected to be a particular threat to larval habitat (Tapley et al., 2017; Nguyen et al., 2020). The species is thought to breed in June, when males vocalise and gravid females have been

collected (Rowley et al., 2013; Nguyen et al., 2020). Tadpoles, associated with gravel substrates, have been observed in September and December, indicating that they may overwinter (Nguyen et al., 2020).

Relatively little is known about the reproductive biology of frogs in the genus *Leptobrachella* (Sung et al., 2021). Whilst the larvae, larval development, and larval habitat of some species are known (e.g. Smith, 1917; Inger, 1985; Malkmus and Kosuch, 1999; Grismer et al., 2004; Ohler et al., 2011; Oberhammer et al., 2014; Hou et al., 2018; Nguyen et al., 2018; Lyu et al., 2020; Nguyen et al., 2020; Le et al., 2021; Shi et al., 2021; Vassiliieva, 2021), breeding seasons are often incompletely known and clutch size and oviposition sites are almost completely unknown. Information on the oviposition sites and clutch size may be important, particularly for threatened species, as such information may inform habitat management, population viability analyses, and population monitoring.

We surveyed suitable habitat within the Hoang Lien Range, to better understand the distribution and natural history of *L. botsfordi*. Herein, we report on the discovery of an important new population of *L. botsfordi* and the first data on oviposition sites and clutch size

Fieldwork was undertaken at a new site south of all previously known localities of *L. botsfordi* on Mount Nam Kang Ho Tao, Hoang Lien National Park, Sa Pa District, Lao Cai Province (22.1505°N, 103.9683°E, 2788 m elevation, Fig. 1, 2A, B) in September 2020 and at a site where *L. botsfordi* has been confirmed previously on Mount Fansipan, Sa Pa District, Lao Cai Province (22.2947°N, 103.8029°E, 2578 m elevation) on 21 June 2021. On 14 September 2020 we encountered eight female *Leptobrachella* (Fig. 2C) that morphologically resembled *L. botsfordi* on Mount Nam Kang Ho Tao. These frogs were found under leaf litter alongside a 150 m stretch of stream flowing through mixed bamboo and rhododendron forest. The stream was small, flowed slowly, and had small pools (Fig. 2B). It was raining during the survey, air temperature was 13.5°C, and ambient humidity was 100% (measured using Kestrel 3500 Delta T Weather Meter, USA). We collected six

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adult female specimens (Vietnam National Museum of Nature, Hanoi, Vietnam, VNMN 2022.001–04; Hoang Lien National Park headquarters, HLNP2020 0914 00027–28), which were euthanised using a 20% solution of benzocaine applied to the ventral surface of the frog. Liver samples were taken for molecular analyses prior to formalin fixation. We examined the ovaries of the preserved specimens and counted ova. Ova measurements were taken with a 30 cm Shenzhen Caister digital calliper.

On Mount Fansipan, we observed several egg clutches. A small number of eggs were collected from one clutch as a voucher (oviposition site 1; Fig. 3A), with several eggs preserved in ethanol; the remaining eggs were preserved in formalin. Developmental stages follow Gosner (1960) and egg measurements were obtained using ImageJ 1.49 (Schneider et al., 2012) from photos of freshly collected eggs prior to preservation, into which a scale had been placed. Voucher eggs were deposited at the Vietnam National Museum of Nature (VNMN 2022.005).

Species identity of the post metamorphic specimens and the eggs was confirmed using molecular analysis of tissue samples from two post metamorphic specimens (VNMN 2022.001 and VNMN 2022.004) and one of the ethanol-preserved egg specimens (VNMN 2022.005) following the methodology of Nguyen et al. (2021). All encountered egg clutches were very similar in appearance and size and were attended by *L. botsfordi*.

We produced a revised species distribution map to assist in the assessment of extinction risk according to the IUCN (IUCN SSC, 2012). The map was created in ArcMap 10.3 using the NASA Shuttle Radar Topography Mission (NASA Shuttle Radar Topography Mission (SRTM), 2013) elevation data at 30 m resolution and ESRI World Imagery basemap layer to determine local land cover. We used 2500 m and 2900 m elevation as lower and upper estimates of elevation range for the species and considered areas isolated from known habitat patches (extant) as potential habitat (possibly extant). Extent of Occurrence (EOO) was measured using the IUCN EOO Calculator tool v1.5 (IUCN, 2023).

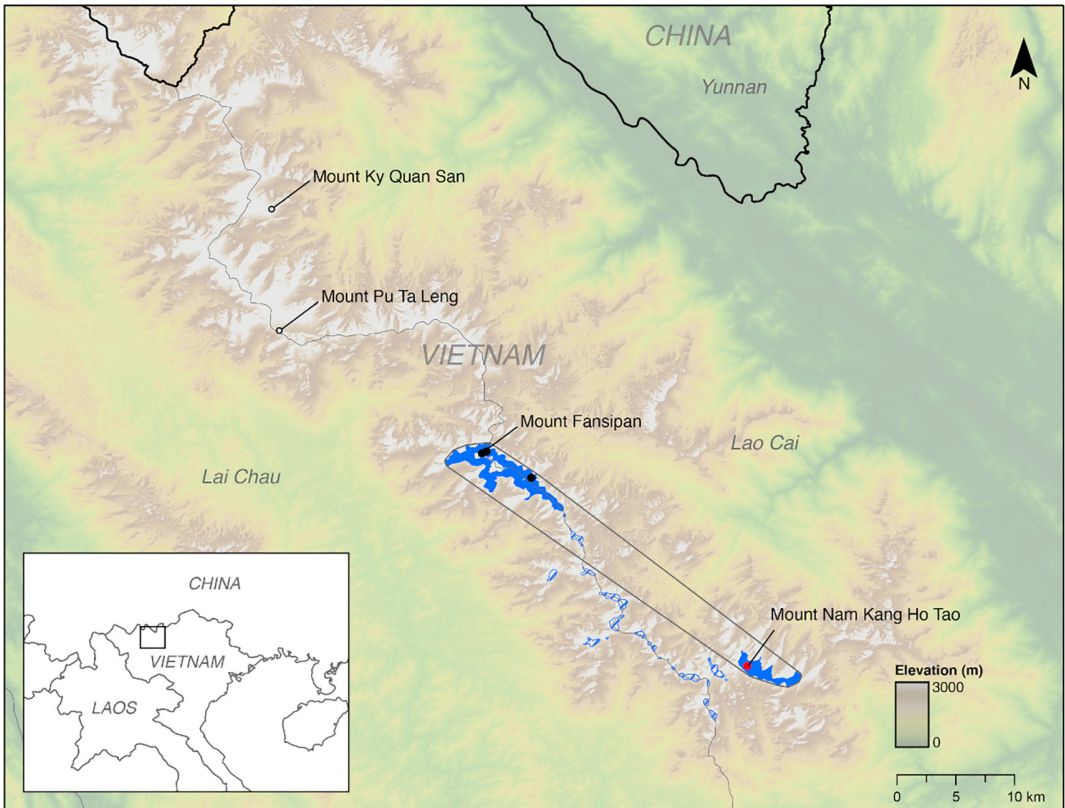


Figure 1. An updated range map of *Leptobranchella botsfordi* in the Hoang Lien Range, Vietnam. New location (red dot), existing known locations (black dots), sites where species were not detected in suitable habitat (white dots), presumed range (solid blue), possibly extant areas (cross-hatched blue), and potential Extent of Occurrence (grey outline).

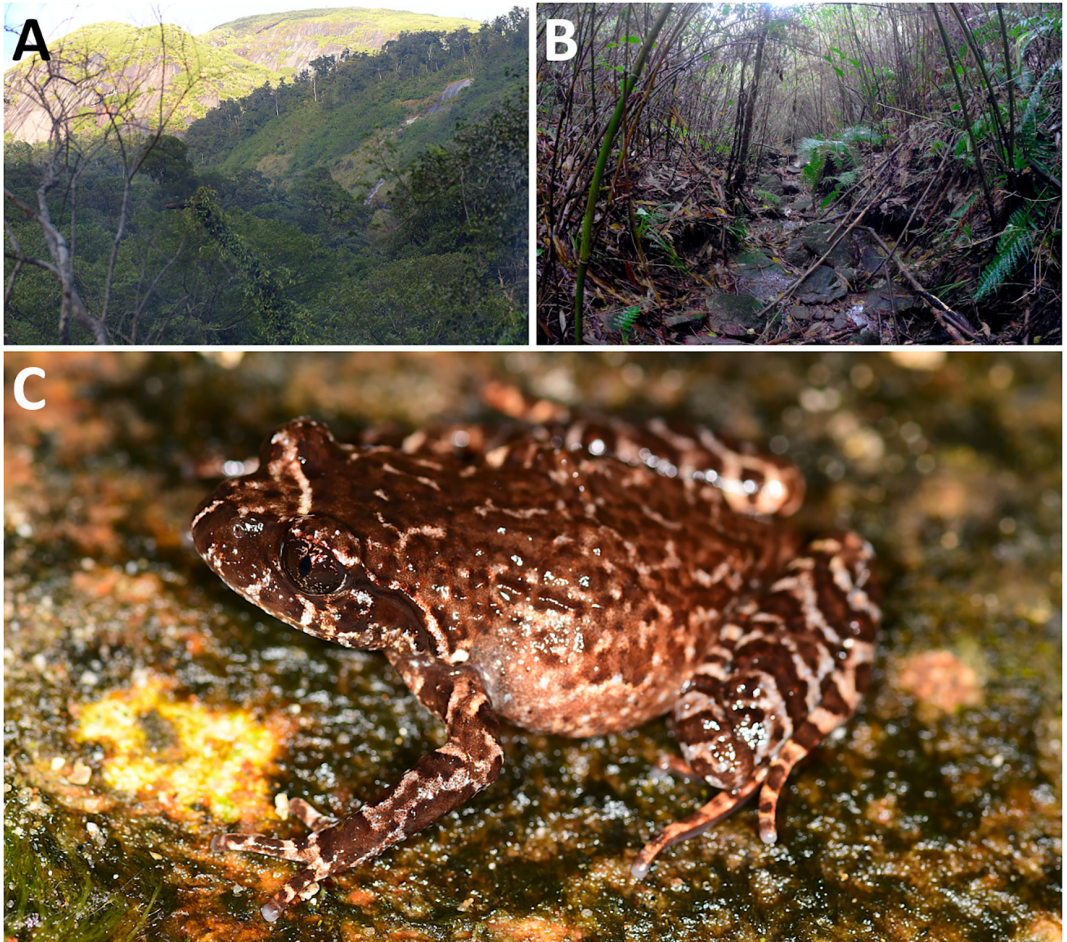


Figure 2. (A) Mount Nam Kang Ho Tao, Sa Pa District, Lao Cai Province, Vietnam; (B) Microhabitat of *Leptobranchella botsfordi*, Mount Nam Kang Ho Tao; (C) Female *L. botsfordi* (VNMN 2022.002) collected here. Photographs by Luan Thanh Nguyen.

The 16S rDNA sequence generated from the two female specimens collected from Mount Nam Kang Ho Tao differed from that of the holotype of *L. botsfordi* (VNMN 03682), which was collected 27 km away on Mount Fansipan (GenBank accession number MH055953.1) by 0.88%. This sequence divergence is congruent with intraspecific variation within the genus (e.g., Nguyen et al., 2018). The new sequences were deposited in GenBank under the accession numbers SUB11849853 and SUB11849855. The new 16S rDNA sequence generated from the egg specimen differed to that of the holotype of *L. botsfordi*, which was collected 3.5 km away on Mount Fansipan (GenBank accession number MH055953.1) by 0.44%. The new sequence was deposited in GenBank under the accession number SUB11849734.

This new site record for *L. botsfordi* on Mount Nam Kang Ho Tao is 23.4 km southeast of the closest reported location of the species on Mount Fansipan, expanding the known Extent of Occurrence for the species from 36 km² to 105.2 km² (Fig. 1). These points are likely two threat-defined locations and it is important that populations at both sites persist in order to conserve genetic diversity and the evolutionary potential of the species. The higher elevations of Mount Fansipan and Mount Nam Kang Ho Tao are the only two relatively large areas of potentially suitable habitat for the species. The small, isolated patches of potentially suitable habitat that exist between Mount Fansipan and Mount Nam Kang Ho Tao may be too small to support viable populations of the species, however further survey work is required to confirm this. It is likely that *L.*

botsfordi qualifies as being reassessed as Endangered in accordance with the IUCN Red List of Threatened Species categories and criteria B1ab(iii), based upon Extent of Occurrence, locations, and ongoing threats. The discovery of a new population of *L. botsfordi* on Mount Nam Kang Ho Tao is particularly important as this site is not currently facing the imminent threat of habitat loss and degradation, particularly gravel mining associated with infrastructural developments for the tourism industry.

On 21 June 2021 we encountered clutches of *L. botsfordi* eggs on Mount Fansipan (22.2947°N, 103.8029°E, elevation 2578 m). At least four clumps of eggs were found among the small roots of bamboo and grasses in a cavity beneath a large, moss-covered boulder, in a seepage, flowing into a small mountain stream (oviposition site 1; Fig. 3A). One of the clutches was adhered to the rocky roof of the cavity. A single *L. botsfordi* of unknown sex was sitting directly on one of the clumps (Fig. 4A, B). It was not possible to estimate the clutch size due to the accessibility of the oviposition site and because we did not wish to disturb the frogs

in case its presence was indicative of parental care. A second oviposition site was found several meters away in another rocky, moss-covered crevice in a seepage (oviposition site 2; Fig. 3B); two individual *L. botsfordi* of unknown sex were present inside the crevice. Only two eggs could be seen within the cavity and as any further investigation of the cavity may have destroyed the oviposition site, we did not examine the site further. Embryos were at stage 1 (Gosner, 1960), suggesting that they had been laid recently. Eggs were a uniform cream colour and surrounded by a fluid filled capsule approximately 0.70 mm wide (Fig. 4C). Egg diameter was highly variable. Mean egg diameter in life was 3.09 mm (2.81–3.45 mm, $n = 7$). This observation confirms that oviposition occurs in June.

We examined the ovaries of six individual *L. botsfordi* collected from Mount Nam Kang Ho Tao and four of them had ova. Ova from four individuals were counted in both ovaries; the average number of eggs was 63 (70 in VNMN 2022.004, 70 in VNMN 2022.002, 61 in VNMN 2022.001, 51 in VNMN 2022.003). The average size of ova in preservative was 1.92 mm (1.5–2.2 mm, $n = 15$).

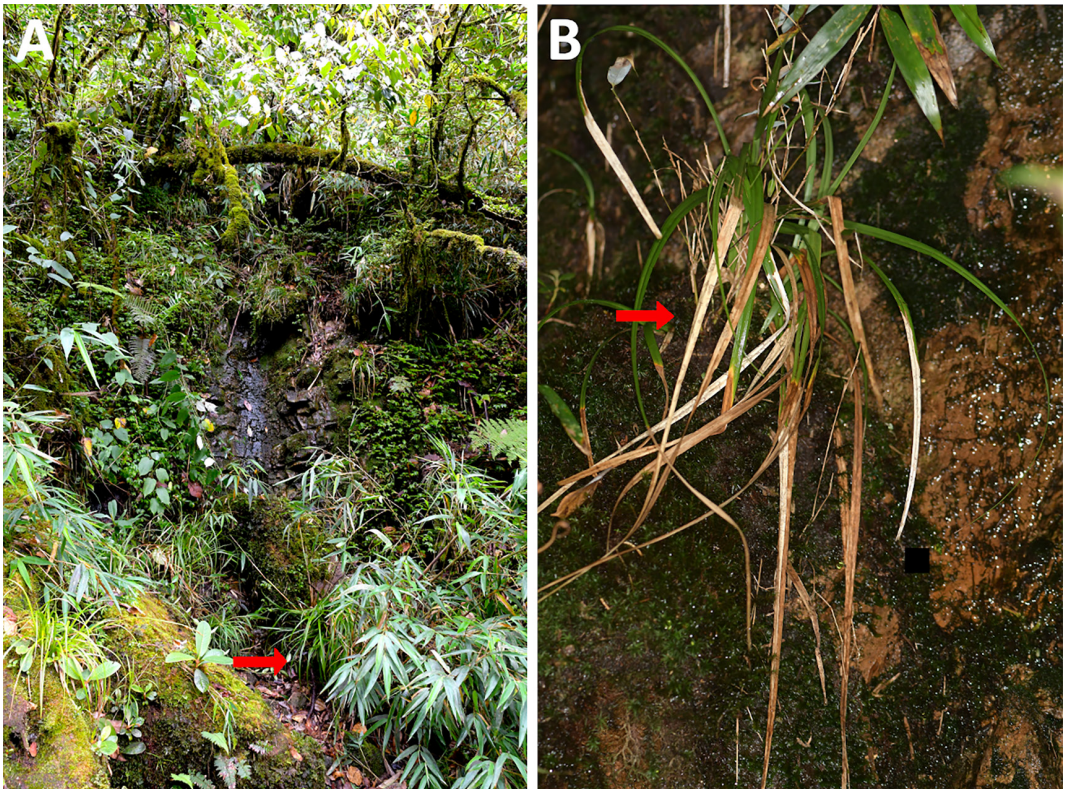


Figure 3. Oviposition sites of *Leptobranchella botsfordi* on Mount Fansipan, (A) site 1 and (B) site 2. Photographs by Luan Thanh Nguyen.

The only published reports on both oviposition sites and clutch size in the genus *Leptobranchella* are from *L. khasiorum* (Das et al., 2010) and *L. tamdil* (Sengupta et al., 2010) in northeastern India. *Leptobranchella khasiorum* is reported to breed in early March and April and adults deposit pale, unpigmented eggs on the underside of rocks that are embedded in puddles in the beds of seasonal streams (Tron et al., 2015). Clutch size ranges from 392–432 eggs and the mean egg diameter was 2.2 mm (Tron et al., 2015); in this study, male frogs were found attending the clutches several days after

they had been laid. *Leptobranchella tamdil* is reported to breed from December to January in the dry season; eggs of *L. tamdil* range between 1.4–1.5 mm and clutch size was 105 eggs (Decemson et al., 2021). Other data on reproductive biology of *Leptobranchella* is in the form of measurements of non-ovulated ova. The left ovary of *L. maurus* (Inger et al., 1997) from montane forests in Borneo contained 45 ova with mean diameter of 1.83–2.33 mm (Inger et al., 1997). *Leptobranchella maurus* had fewer, larger ova relative to other *Leptobranchella* species in Borneo and it is suggested that larger ova

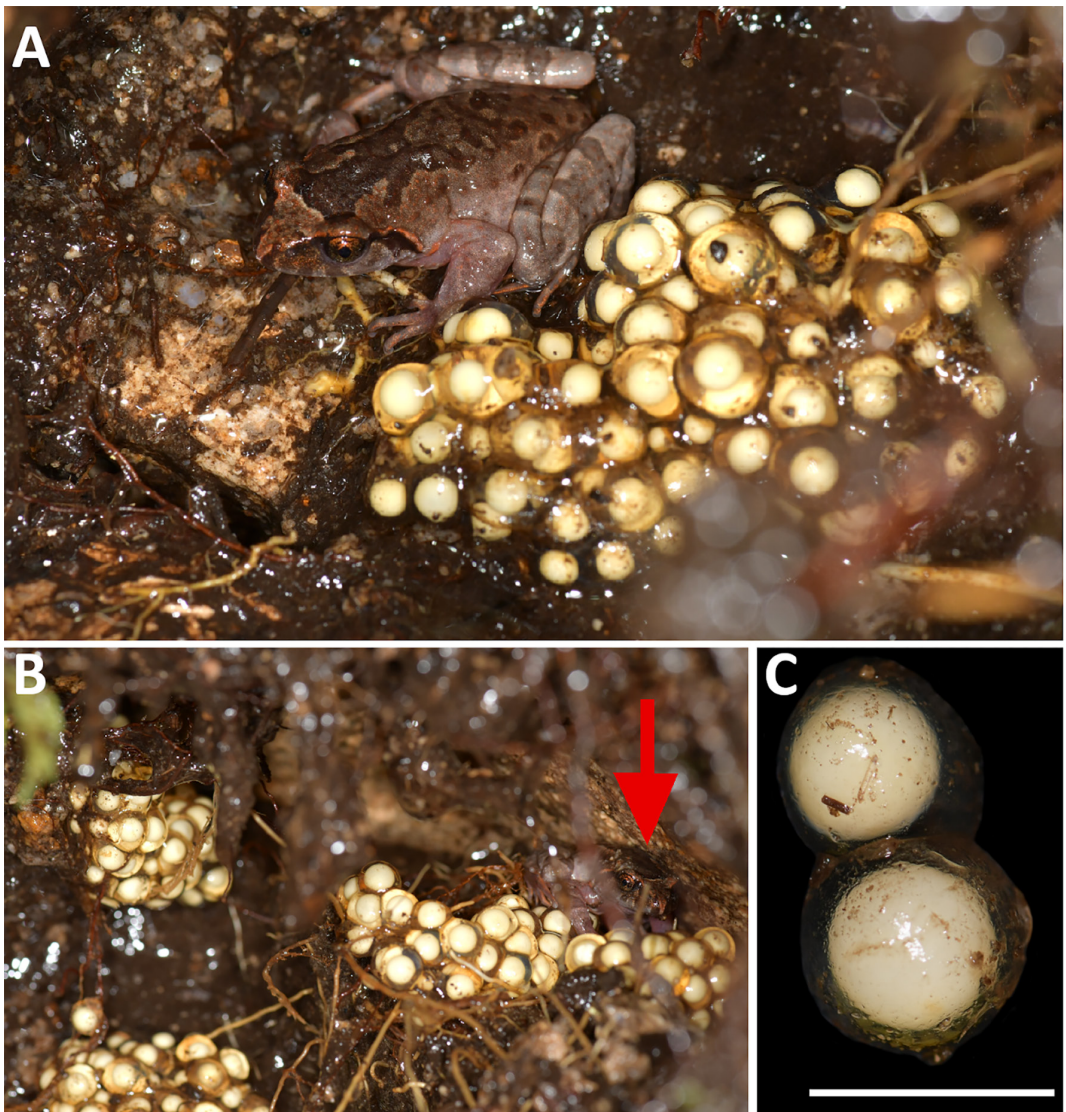


Figure 4. *Leptobranchella botsfordi* clutches and eggs from Mount Fansipan, Lao Cai Province, Vietnam. (A) Adult sitting beside egg clutch. (B) Several clutches of eggs (Gosner Stage 1) with an adult sitting on top of a clutch (red arrow; same individual as in A). (C) Two eggs, Gosner Stage 1 (VNMN 2022.005). Scale = 5 mm. Photographs by Luan Thanh Nguyen.

are typical of frogs associated with higher elevation in Borneo (Inger et al., 1997).

These are the first data on the eggs and oviposition sites of *Leptobranchella* in Vietnam and further surveys are required to confirm the clutch size of this species. The diameter of freshly laid eggs (up to 3.45 mm) is the largest reported so far for this genus; eggs are 36.2% larger than those laid by *L. khasiorum* and 42.0% larger than those laid by *L. tamdil* (Tron et al., 2015; Decemson et al., 2021). The egg diameter of some frogs is known to vary in response to dehydration (Salica et al., 2017) and parental attendance (Bickford, 2004) and so direct comparisons of egg size are difficult. Our observations of frogs in close proximity to eggs warrants further research into potential egg attendance and possible parental care in the genus *Leptobranchella*.

Previous work has highlighted the need to preserve gravel beds, likely an important larval microhabitat for *L. botsfordi* (Nguyen et al., 2021). Our recent finding indicate that this species also requires rocky seepages for oviposition. The loss of any microhabitats required by this species at different life stages, or during breeding events, may be detrimental to population recruitment. We recommend that the new location on Mount Nam Kang Ho Tao and the riparian vegetation including oviposition sites on Mount Fansipan are strictly protected given that the habitat at the only other known locality of this species is already severely degraded. Despite intensive searches, larvae and eggs have never been found at the only other known site. Further research is needed to determine habitat use outside the breeding season.

Prioritising species and habitats for conservation is dependent on the knowledge of the distribution and biology of amphibians (Rowley et al., 2010). Our study further highlights the importance of basic amphibian inventory surveys and taxonomic work in the Hoang Lien Range. Recently, the published range extension of *Oreolalax sterlingae* Nguyen et al., 2013, resulted in this species being downlisted from Critically Endangered to Endangered (Tapley et al., 2020; IUCN SSC, 2021b). It is vital that such work continues to ensure that the limited resources for conservation are used effectively.

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