REPTILES & AMPHIBIANS

A Hibernaculum of Two Rhacophorid Species from India

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Frogs are ectotherms that must brumate during cold, dry months in hibernacula that provide the appropriate physical and microclimatic characteristics (Swanson and Burdick 2010); these include optimum temperature, consistent oxygen and humidity levels, low-intensity short-photoperiod lighting, and minimum physical disturbance (Browne 2007). Avoidance of water loss is the main reason frogs brumate in burrows (Ruibal et al. 1969), and even treefrogs brumate in soil to avoid desiccation during the dry season (Ihara 1999). Depending on their preferred natural habitat, brumating frogs seek winter refugia under water or in burrows in moist soil, hollows of logs, cracks and crevices of rocks, and in leaf litter (Annandale 1912; Cunjak 2011; Borzée et al. 2018, 2019).

Among treefrogs of the family Rhacophoridae, Schlegel's Green Treefrog (*Zhangixalus schlegelii*), which is endemic to Japan, is known to brumate in soil (Ihara 1999). This also has been observed in the Giant Treefrog (*Z. smaragdinus*), which is commonly found in northeastern India (B. Saikia, pers. obs.). Interestingly, the latter undergoes a color change from green to brown when brumating in soil. The type series of the Black-spotted Frog (*Theloderma moloch*) was collected from underneath a log during the dry winter months in the present-day state of Arunachal Pradesh, India (Annandale 1912), and the type series of the Shillong Bush Frog (*Raorchestes shillongensis*) was found in soil during an earth cutting in Meghalaya, India (Pillai and Chanda 1973). Although, this species is known to have vibrant color morphs (Baruah et al. 2018), Pillai and Chanda (1973) reported that the color of the frogs blended perfectly with the color of the earth, suggesting that they too undergo color changes during brumation. Interestingly, we have observed beige-brown *R. shillongensis* brumating in hollow logs at the type locality. Among other treefrogs in the region, Iangrai (2011) noted that *Rhacophorus bipunctatus* "hibernates" in tree holes and on banana stems. However, beyond these sporadic reports, very little is known about brumation in other rhacophorids in the region.

On 14 February 2017, a rhacophorid (*Raorchestes* sp.) was collected from a banana plant at Malidor Village in Cachar District, Assam, in northeastern India. Taking a cue from this experience, on 31 January 2019, we searched for frogs in a banana plantation near Murlen Village, Mizoram, India (23.662778°N, 93.28639°E; elev. 1,394 m asl) (Fig. 1) — we found frogs within the inner leaf-sheath layers of banana plants when we peeled off the outer leaf-sheath. The frogs were resting with eyes closed and limbs tightly adpressed; however, when disturbed, they instantly tried to retreat into the interior of the leaf-sheath where a small amount of water was present. We observed snails, slugs, earthworms, earwigs,

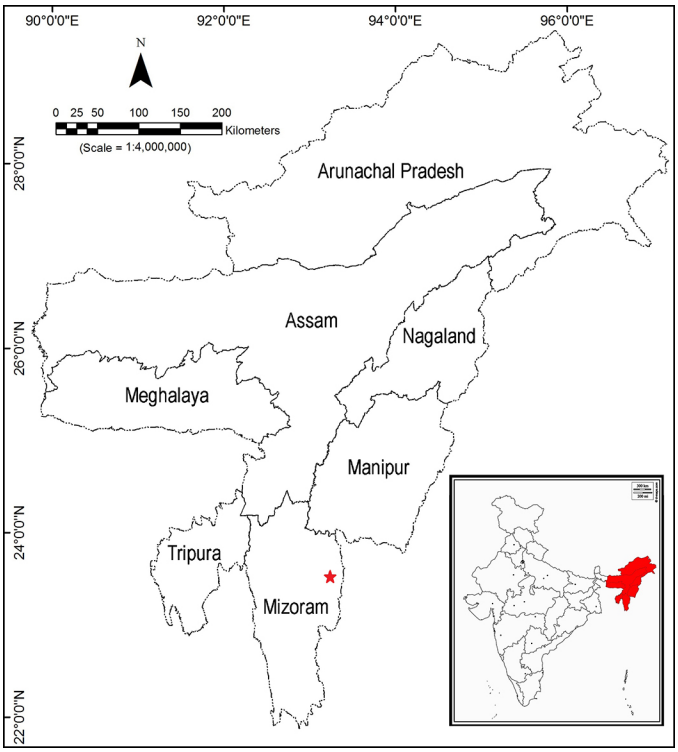

Fig. 1. Map showing the location of Murlen Village in Mizoram, Northeast India. Inset: Map of India, with seven Northeast Indian states highlighted in red.

and other invertebrates occupying this same space. Even during cold, dry winter conditions, the banana pseudostem retains sufficient moisture to provide a warm and moist environment with more or less constant climatic condition — making it an ideal hibernaculum for brumating treefrogs.

We collected eight frogs and deposited specimens in the museum of the Zoological Survey of India (ZSI), Shillong, two (V/A/NERC/ZSI/1499, n = 2) that we believed to be Jerdon's Bush Frogs (*Nasutixalus jerdonii*) and six (V/A/NERC/ZSI/1498, n = 5; V/A/NERC/ZSI/1498, n = 1) that we believed to be Boulenger's Bush Frogs (*Kurixalus verrucosus*).

The specimens that we attributed to *N. jerdonii* (Figs. 2A–B) had morphological features typical of the species. Both were small (♂ SVL 27.19 mm; ♀ SVL 41.46 mm) with a truncated snout, dark stripes from the snout to the anterior cor-

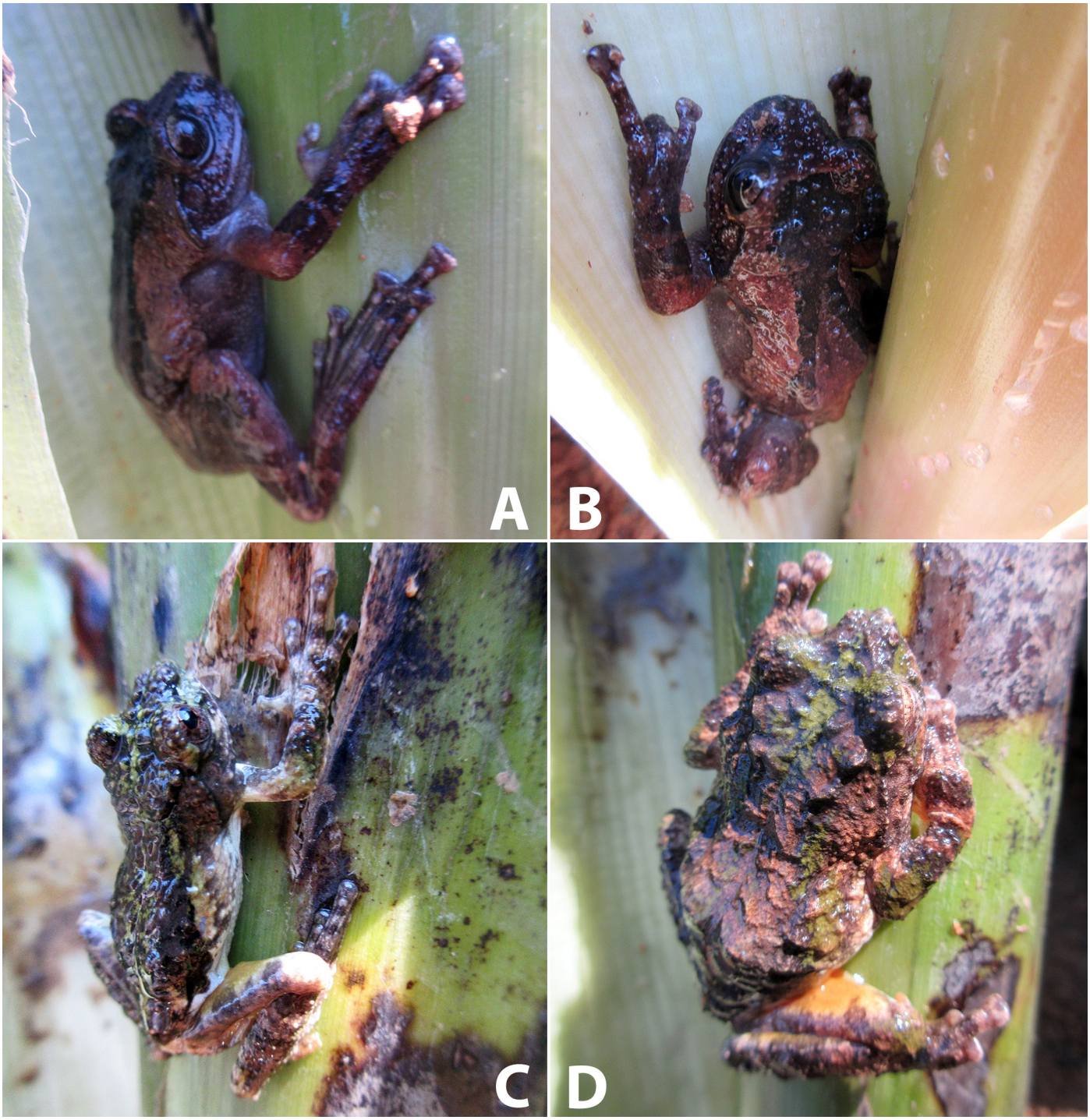

Fig. 2. Jerdon's Bush Frogs (*Nasutixalus jerdonii*), male (A) and female (B), and Boulenger's Bush Frogs (*Kurixalus verrucosus*), male (C) and female (D), from Murlen Village, Champhai, Mizoram, India. Photographs by Bhaskar Saikia.

ners of the eyes, dorsum brownish-gray with a large X-shaped mark, flanks light yellow, limbs banded, toes half webbed, and fingers free. This species was described by Günther (1876) from the "Darjeeling Hills" of West Bengal, India, but was not seen again until 140 years later when Biju et al. (2016) rediscovered the species from the northeastern Indian states of Meghalaya, Manipur, and Nagaland. Soon after, Lalronunga et al. (2017) reported this species from Aizawl District of Mizoram; our frogs from Murlen are from adjacent Champhai District and represent a new district record. Recently, a single specimen of *N. jerdonii* was reported by Rahman et al. (2020) from Myanmar, based on molecular studies; however, morphologically, that specimen differs from a typical *N. jerdonii* in not having a truncated snout and in lacking an X-shaped mark on the dorsum, which the authors attributed to geographic variation. Another report of *N. jerdonii* from Bhutan by Wangyal et al. (2020) could not be confirmed as conspecific from their report. Interestingly, Pawar and Birand (2001) had reported the occurrence of "*Rhacophorus* cf. *jerdoni*" from Mouling National Park in Arunachal Pradesh, India, but without a voucher specimen. Although the morphological characters mentioned by them do agree with the generic characters of *Nasutixalus,* the report of a dull-green dorsum deviates markedly from the brownish-gray of a typical *N. jerdonii*. Notably, Biju et al. (2016) in their paper on the rediscovery of the species, did not refer to this "sighting."

The specimens that we attributed to *K. verrucosus* (Figs. 2C–D) also had morphological features typical of the species. These were medium-sized frogs (♂ SVL 22.6–25.8 mm, n = 4; ♀ SVL 45.2–45.9 mm, n = 2) with a pointed snout, nostrils near the snout, tympanum distinct and half the diameter of the eye, vomerine teeth in two short rows and touching the choanae anteriorly, fingers webbed at the base and ending in disks about the size of the tympanum, toes entirely webbed and with single well-developed subarticular tubercles, inner metatarsal tubercles small, tibio-tarsal articulation extending to between the eye and nostril, dorsum gray or brown with small warts and marbled with darker shades, venter pale and granulated, and limbs with irregular bands. This species was described by Boulenger (1893) from Myanmar. The first report of this species in India was by Ohler et al. (2018), who reported it from Anini in Arunachal Pradesh. The species also is known from China, Myanmar, Thailand, and Vietnam (Frost 2021). This report extends the range of *K. verrucosus* into Mizoram, India, and also represents the westernmost record of the species.

To confirm our morphologically based identifications of these frogs, we verified their identities using a 16S rRNA gene marker-based genetic kinship test. We excised liver tissues from one *N. jerdonii* and two *K. verrucosus* for genomic DNA extraction (Al-janabi and Martinez 1997). The quality of DNA was assessed by agarose gel electrophoresis and by A260/A280 ratio. We used the PCR primers 16sar-L (5'CGCCTGTTTATCAAAAACAT3') and 16sbr-H (5' CCGGTCTGAACTCAGATCACGT3') of Palumbi et al. (2002) to amplify 16S rRNA gene fragments (697 bp) from the genomic DNA of the samples. Sanger sequencing of amplicons were outsourced. The chromatogram and FASTA files received from the sequencing service provider were utilized for trimming low-confidence base calls (Phred score <20) from the ends. The edited sequences were submitted to NCBI GenBank (accession numbers MN519708.1, MT632252.1, and MN519706.1). Twenty-eight GenBank sequences with 90–100% query cover and 94–100% sequence identity with our three sequences were retrieved and incorporated into a phylogenetic analysis. The data sets comprising 31 16S rRNA gene sequences were aligned according to the MUSCLE algorithm using MEGA X (Kumar et al. 2018), and a consensus maximum-likelihood neighbor-joining phylogenetic tree (Fig. 3) was built on the GTR + G (General Time Reversal and Gamma Distribution) nucleotide substitution model because it had the lowest BIC (Bayesian Information Criterion) score. We performed 500 bootstrap replication runs to obtain the consensus tree.

Our *Nasutixalus jerdonii* sample (MN519708.1) is in cluster I with low bootstrap separation from *N. jerdonii* vouchers KU170001.1, KU170003.1, KU169996.1, and KU170000.1. Our *Kurixalus verrucosus* (MN519706.1 and MT632252.1) samples are grouped in cluster III with 12 other *K. verrucosus* vouchers. These results verify the identities of our samples. Interestingly, three sequences (MT742623.1, MT741785.1, and MT742622.1), labeled in GenBank as "*Kurixalus yangi*," need to be reevaluated as they fell within the sequences of *K. verrucosus* used in this study.

Perusals of the literature on brumation of frogs yielded no references to the use of banana leaf-sheaths as hibernacula, other than the passing remark by Iangrai (2011) on the use of a banana stem for "hibernation" by *Rhacophorus bipunctatus*. The use of banana leaf-sheaths for brumation by *N. jerdonii* and *K. verrucosus* is not surprising, as the conditions in the sheaths are suitable; also, species of *Nasutixalus* are known to breed in phytotelmata (Biju et al. 2016; Yang and Chan 2018). Also of interest is that, unlike *Z. smaragdinus* and *R. shillongensis*, which change color during brumation, we observed no color changes in brumating *N. jerdonii* and *K. verrucosus*.

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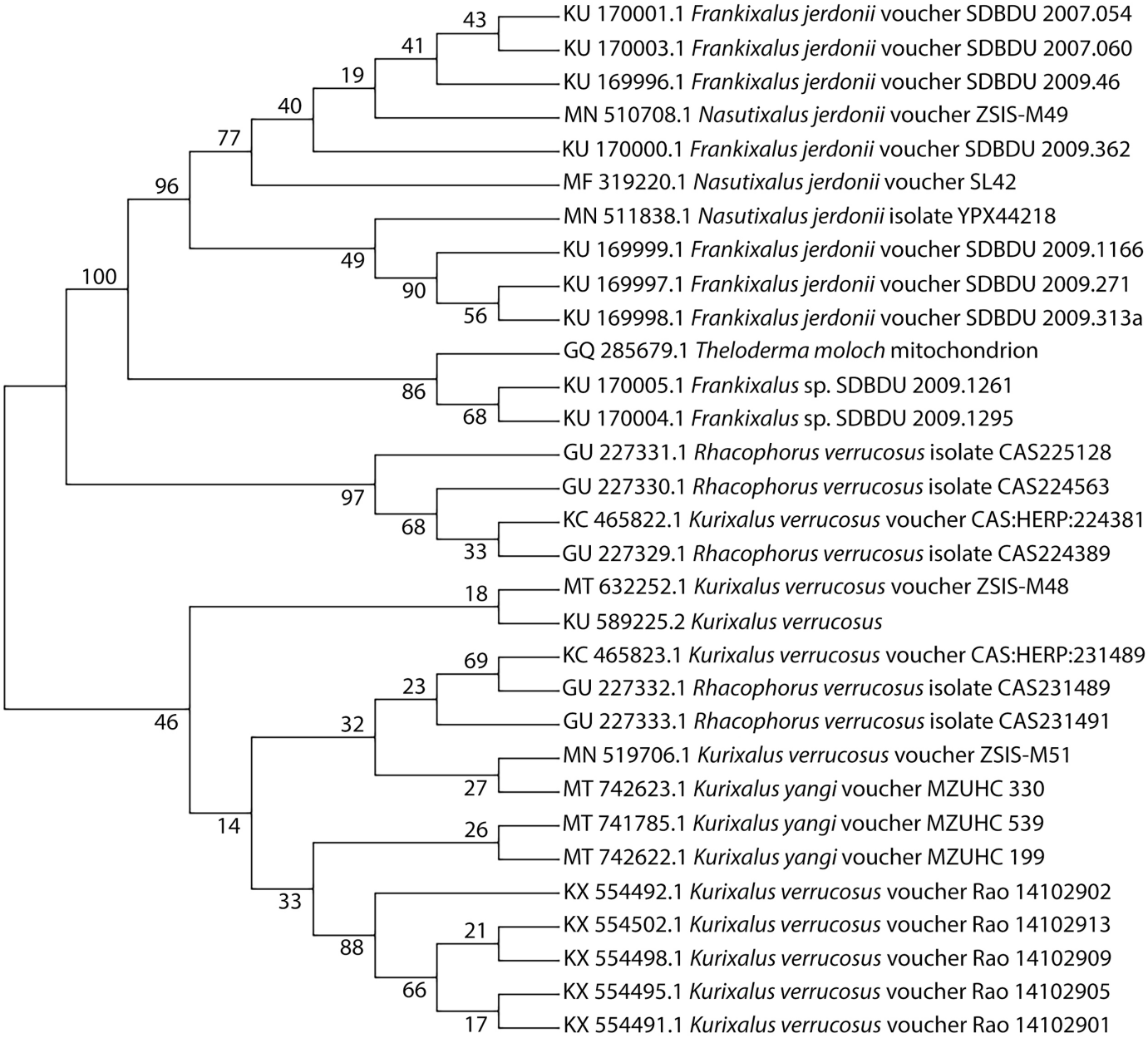

Fig. 3. Consensus maximum-likelihood neighbor-joining tree depicting genetic relationships among Jerdon's Bush Frogs (*Nasutixalus jerdonii*) and Boulenger's Bush Frogs (*Kurixalus verrucosus*). Figures on nodes are bootstrap support values obtained from 500 replications.

Molecular genetics work was done in the DBT-BIF of St. Anthony's College, Shillong, Meghalaya, India.

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