



A new species of the genus *Nasikabatrachus* (Anura, Nasikabatrachidae) from the eastern slopes of the Western Ghats, India

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We describe a new species of the endemic frog genus *Nasikabatrachus*, from the eastern slopes of the Western Ghats, in India. The new species is morphologically, acoustically and genetically distinct from *N. sahyadrensis*. Computed tomography scans of both species revealed diagnostic osteological differences, particularly in the vertebral column. Male advertisement call analysis also showed the two species to be distinct. A phenological difference in breeding season exists between the new species (which breeds during the northeast monsoon season; October to December), and its sister species (which breeds during the southwest monsoon; May to August). The new species shows 6 % genetic divergence (K2P) at mitochondrial 16S rRNA (1330 bp) partial gene from its congener, indicating clear differentiation within *Nasikabatrachus*. Speciation within this fossorial lineage is hypothesized to have been caused by phenological shift in breeding during different monsoon seasons—the northeast monsoon in the new species *versus* southwest monsoon in *N. sahyadrensis*. It is postulated that proximate triggers of breeding behavior and highly stenotopic adaptation of *Nasikabatrachus* tadpoles to inhabit cascades during monsoonal stream flows, have led to allopatry on the eastern and western slopes of the Western Ghats, thereby promoting speciation in this ancient genus.

<http://zoobank.org/urn:lsid:zoobank.org:pub:56A35631-4676-4899-8EB4-7C2BBFB24223>

INTRODUCTION

The family *NASIKABATRACHIDAE* was established in 2003 with a monotypic genus represented by *Nasikabatrachus sahyadrensis* (Biju & Bossuyt 2003), collected from the secondary forest at Kattappana, Idukki district, Kerala, Western Ghats mountains, India. Molecular evidence presented with its description showed the family to be the sister-taxon of the family *SOOGLOSSIDAE* Noble, 1931, at least among the extant fauna. The latter family is represented by two genera, *Sooglossus* Boulenger, 1906 and *Sechelophryne* Nussbaum & Wu, 2007, each encompassing two species, found in the Seychelles Islands in the Indian Ocean. The superfamily *SOOGLOSSOIDEA* Noble, 1931 (*sensu* Dubois 2005) is hypothesized to have originated during late Jurassic or early Cretaceous period (180–160 mya) and *SOOGLOSSIDAE* and *NASIKABATRACHIDAE* to have diverged from each other around 120–80 mya ago (Bossuyt & Roelants 2009) by vicariance, as the landmasses of the Seychelles and the Indian Plate of Gondwana separated and drifted away during the Paleocene epoch. Since the description of this Gondwanan relict frog, there have been several reports from various locations within the Western Ghats, increasing its known distribution range

(Aggarwal 2004; Dutta *et al.* 2004; Das 2006; Radhakrishnan *et al.* 2007; Jobin *et al.* 2012). The known range of *N. sahyadrensis* lies within the bounds of 9.03–11.26°N and 76.12–77.65°E. This frog is recorded from elevations ranging from 50 to 1100 m a.s.l., occupying the habitats from deciduous forests to moist evergreen forests (fig. 1). However, all reports on occurrence of *N. sahyadrensis* known to date are from the western slopes of the Western Ghats (fig. 1), with breeding time at the onset of southwest monsoon (starting late May, and lasting until September). *Nasikabatrachus sahyadrensis* is a fossorial species, having only a very brief period of terrestrial activity, being observed above ground only during the breeding season, and as larvae in streams (Dutta *et al.* 2004; Raj *et al.* 2011).

During our recent field visits, we first noted the presence of *Nasikabatrachus* sp. by sighting its distinctive tadpoles in a stream that flows from the eastern slopes of the Western Ghats, during the northeast monsoon in 2010. We used morphological, molecular, and bioacoustics data to ascertain the species status of this population. As a result, we describe a new species of the genus *Nasikabatrachus* that occurs on the eastern slopes of the Western Ghats.

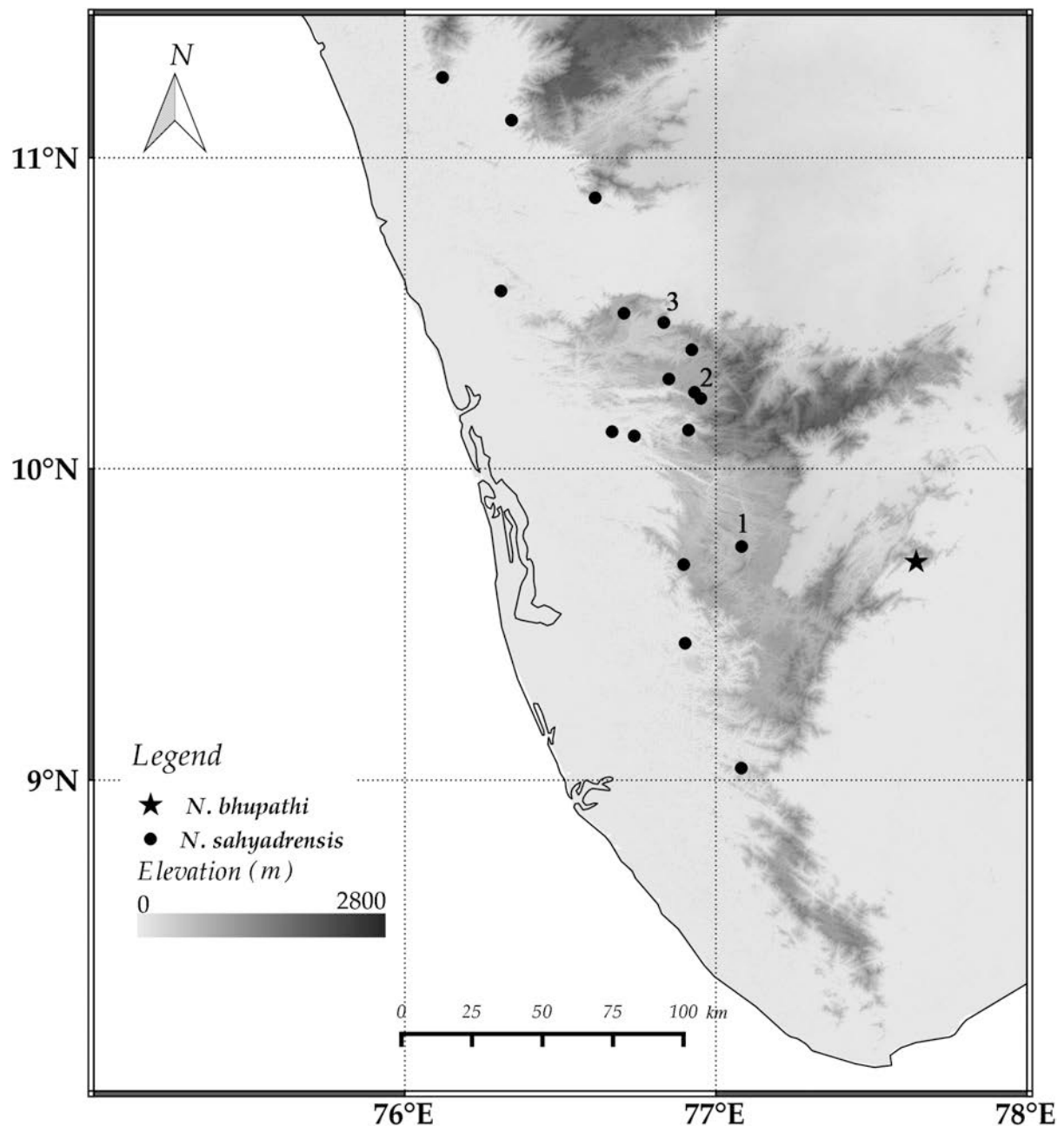


Figure 1. Distribution of the Indian endemic family *NASIKABATRACHIDAE* in the Western Ghats Mountains, southwestern India. Circles: *Nasikabatrachus sahyadrensis* (1: type locality, 2: Sankaran Kudi, Dutta *et al.* 2004, 3: Karean shola, Anamalais, Raj *et al.* 2012); star: *Nasikabatrachus bhupathi*, type locality.

MATERIALS AND METHODS

Specimen collection and preservation

We detected the presence of populations of *Nasikabatrachus* sp. thanks to their mating calls from burrows along the ephemeral stream flowing through a farm adjacent to the Watrap Range of the Srivilliputhur Grizzled Giant Squirrel Wildlife Sanctuary (SWS) in 2012. We collected two adult specimens (ZSIA 14153 and ZSIA 14174) from an agricultural field near the forest edge, outside of the Watrap Range of the SWS, at an elevation of 200 m a.s.l. (fig. 1), while they were calling. We do not provide the coordinates on the locality as it falls within privately owned areas and we comply with the request made by the land owners. The area primarily receives rainfall during the northeast monsoon (October to December) and most of the streams dry up as the summer approaches. The forest types at the type locality are southern dry mixed deciduous forest and Carnatic umbrella thorn forest (fig. 2) (Champion & Seth 1968). We used 10 % formalin to fix and preserve one of the adult specimens and 70 % ethanol to preserve the other after collecting tissue samples for genetic analysis. Six tadpoles (ZSIA 14158–14163) were also preserved for both genetic and morphometric analysis, as well as four imagos (just metamorphosed froglets; see Vences 2004) (ZSIA 14154–14157) raised by us, under semi-natural conditions, from tadpoles that we had collected in advanced stages of development (stage 38 and above, Gosner 1960). We raised them in stream water stored in earthen pots, away from the stream where they had been found. We fed them a combination of boiled and minced spinach, algal scrapings from the breeding sites and chicken egg yolk, coated on the rocky substratum and on the walls of the earthen pots in which they were raised.

Acoustic characterization

We recorded the male advertisement calls of the new species *in situ* during the breeding season using a Nikon S4 digital camera, as audio file in 8 bit format. Recordings were analyzed with RAVEN Lite v1.0 (Cornell Bioacoustics Research Group) and compared with the call patterns of *Nasikabatrachus sahyadrensis* from Macaulay Library of Natural Sounds Catalogue No. 163897 and Thomas *et al.* (2014). Calls were digitized at a sampling rate of 7872 Hz for *Nasikabatrachus* sp. and 48000 Hz for *N. sahyadrensis*, respectively. We used the following setting to produce the sonograms for both the species: Discrete Fourier Transform (DFT) size = 256 samples, frequency grid size = 30.75 Hz, time grid size = 16.2 ms for *Nasikabatrachus* sp.; and DFT = 256 samples, frequency grid size = 187.5, time grid size = 2.67 ms for *N. sahyadrensis*. Descriptors of call parameters are given as means and two standard deviations.

Morphometrics

We obtained the following measurements from the preserved specimens (two adult males and four imagos of *Nasikabatrachus* sp.) and compared them with the measurements given in Dutta *et al.* (2004) for *N. sahyadrensis*: snout-vent length (SVL); eye diameter (ED); inter narial distance (IND); inter orbital distance (IOD); tibia length (TL); inner metatarsal tubercle length (IMT); and inner metatarsal tubercle width (IMTW). All measurements were made with digital calliper to the tenth of millimeter (Table 1). We followed Gosner (1960) for staging the tadpoles and Raj *et al.* (2012) for description of larval external morphology.

Computed tomography

We used the computed tomography (CT) scanning facility at the Indian Institute of Technology, Kanpur to examine osteological characteristics in a representative adult male of *N. sahyadrensis* (KAUNHM 2011131, SVL = 57.1 mm; collected by Jobin K. M. and P. O. Nameer on 11 June 2011 at Pattikkad, Peechi Forest Division, Thrissur, Kerala) and the adult male (ZSIA 14153) of *Nasikabatrachus* sp., which was destined to be the holotype. We used a CT-MINI desktop scanner (Procon X-ray GmbH) with a microfocus X-ray tube (7-micron focal spot) and a Hamamatsu flat panel detector (1024 × 1024 photo-diodes). We set the resolution of the scans at 60 $\mu\text{m}/\text{voxel}$ for *Nasikabatrachus* sp. and at 65 $\mu\text{m}/\text{voxel}$ for *N. sahyadrensis*. Data were exported as .TIFF image stacks in the original 8-bit imagery. We imported the data into the volumetric rendering program VG Studio MaxE v2.2 (<http://www.volumegraphics.com>) at the Microscopy and Imaging Facility, AMNH, New York (USA). Images of the whole skeletal views were produced from the CT scans in VG Studio MaxE v2.2. Images were further processed in Adobe Photoshop CS5. We followed Scott (2005) to describe the characters. Presacral Vertebrae (PSV) were



Figure 2. Habitat of *Nasikabatrachus bhupathi*. (a) vegetation at the type locality; (b) typical breeding habitat of *N. bhupathi*; (c) stream bed where specimens of the type series were collected.

numbered in the series and abbreviated as PSV x , where x is the number. As the first two sacral vertebrae are fused in *Nasikabatrachus*, the first counted vertebra is referred to as PSV1+2, and it bears one set of transverse processes.

We focus here on whether there are any differences between our putative new species and *N. sahyadrensis*. The general osteology of *Nasikabatrachus* is not addressed here and is beyond the scope of this publication. We are aware of the limitations of comparing the osteology of only one adult male of each taxon via CT scans. If even present, osteological differences are usually subtle between pairs of sister-species. We do not report on subtle differences found in the appendicular skeleton, as these may be more highly dependent on the degree of ossification than those in the axial skeleton. Our two specimens differed in degree of ossification, which may be age related, or be species dependent. The differences discovered are considered relevant, because gross osteological differences between closely related taxa, such as sister-species pair, are seldom encountered in Anura (Nussbaum & Wu 1997, Wu 1994). We do not report on subtle differences found in the appendicular skeleton, as these may be more highly dependant on the degree of ossification than those in the axial skeleton. Intra-specific (populational) variation in osteology is poorly studied, but what research has been conducted (Trueb 1977) has shown that although osteology may differ markedly between semaphoronts, it does not usually differ much between adult individuals within a semaphoront class. We examined the osteology of adult males. Differences between the newly discovered species

Table 1. Morphometrics of adult and imagos of *Nasikabatrachus bhupathi*.

Measure- ments	<i>Nasikabatrachus sahyadrensis</i>		<i>Nasikabatrachus bhupathi</i> sp. nov.					
	BNHM 4214	KAUNHM 2011131	Holotype	Paratypes				
	Male	Male	Adult Male	Adult male	Imagos			
			ZSIA 14153	ZSIA 14174	ZSIA 14157	ZSIA 14154	ZSIA 14156	ZSIA 14155
SVL	52.8	57.1	48.5	45.9	21.1	22.5	17.3	17.2
ED	2.8	2.8	2.3	2.3	1.1	1.3	0.9	1.0
IND	3.3	3.5	2.5	2.1	1.4	1.5	1.2	1.2
IOD	8.3	8.8	8.0	7.7	4.7	4.2	3.9	4.1
TL	22.7	21.4	18.7	17	6.7	6.7	5.1	5.5
IMTL	8.8	8.5	7.5	6.2	2.6	2.7	2.1	1.9
IMTW	3.9	3.3	3.2	2.5	1.1	1.2	0.9	0.8

and *N. sahyadrensis* in characters that traditionally only differ at the higher systematic level, are presented in the results section. We found further subtle differences in the pectoral girdle, but as the might be due to the degree of ossification, they are omitted from the description.

Molecular methods

We isolated total genomic DNA from the ethanol-preserved tissue samples using the phenol-chloroform extraction method, after rehydration with saline phosphate buffer (Dutta *et al.* 2004). Partial mitochondrial 12S and 16S rRNA gene fragments were amplified using published as well as newly designed primers. The primers used for PCR are: 12S_trnaf_ranL (5'GCRCTGAAAACGCTAARATGRACCC3', this study) and 12S_1009_anuH (5' CTTACCRTGTTACGACTTRCCTCTTC3', this study) for 12S rRNA gene; and 16S_230_uniL (5'AGTACCGCAAGGGAAIIRTGAAATA3', this study) and 16SbrH (5' CCGGTCTG-AACTCAGATCACGTA3', Palumbi *et al.* 1991) for 16S rRNA gene. DNA amplification, amplicon purification and sequencing were done as described in Shanker *et al.* (2004). We used the following internal primers to sequence the amplicons bi-directionally: 12SAL (5'AAACTGGGATTAGATACCCCACTAT3', Palumbi *et al.* 1991), 12SeL (5'GGGAAGAAATGGGCTACATTTTCT3', Cannatella *et al.* 1998) for 12S rRNA gene; 16SL10 (5' AGTGGGCCTAAAAGCAGCCA3', Hay *et al.* 1995) and 16SarL (5' CGCCTGTTTACCAAAAACAT-CGCCTC3', Palumbi *et al.* 1991) for 16S rRNA gene. These amplicons were blasted against the database sequences and compared with the available sequences of *Nasikabatrachus* and sooglossids, by calculating the pairwise K2P (Kimura 1980) genetic distances between them using MEGA 6.0 (Tamura *et al.* 2013).

Molecular phylogeny

We constructed a molecular phylogeny based on the partial 12S (824 bp) and 16S (1330 bp) rRNA gene along with sequences from *NASIKABATRACHIDAE*, *SOOGLOSSIDAE*, and representatives of archeobatrachian and neobatrachian frog families. Details of the sequences (along with GenBank accession numbers) used for the phylogenetic analysis are given in Table 2. We aligned and manually edited all sequences using CLUSTALX 2.0 (Thompson *et al.* 1997), inferred the best fitting DNA substitution model using the Akaike Information Criterion (AIC) as implemented in jModelTest2 (Guindon & Gascuel 2003, Darriba *et al.* 2012). Phylogenetic analysis was conducted using both maximum likelihood (ML) and Bayesian inference (BI) methods. We used *Leiopelma archeyi* as the outgroup in our analysis. BI was implemented in MrBayes v3.2 (Ronquist *et al.* 2012) using the following parameters: GTR+G+I model of DNA substitution; Nst = 6 (all different substitution rates subjected to GTR); flat dirchlet prior for both substitution rates and the stationary nucleotide frequencies of the GTR rate matrix, a uniform distribution (0.1) for both; the shape parameter of the gamma distribution of rate variation and the prior for the proportion of invariable sites. We performed 2,000,000 MCMC iterations in two runs and four chains, with

sampling in every 300 iterations. We set the minimum standard deviation of the split frequencies as 0.01 and discarded the initial 25 % of stored trees and parameters as burn-in. Similarly, ML analyses were implemented in RAxML v7.2.6 (Stamatakis 2006) with GTR+G model of DNA substitution. We performed 100 replicates to obtain the best-scoring ML tree and 500 bootstrap iterations to obtain clade support values. To understand the molecular differentiation among the western and the eastern populations of *Nasikabatrachus* sp., we performed a separate phylogenetic analysis (combined 12S and 16S partial rRNA gene) with all available sequences of *Nasikabatrachus* sp. and representative sequences of *SOOGLOSSIDAE*, with *Heleophryne purcelli* as outgroup.

Table 2. List of taxa included in the molecular phylogenetic analysis presented.

Taxon	Accession No.	
	for phylogeny	for K2p genetic distances
<i>Amolops tormotus</i>	DQ835616	-
<i>Amnirana albolabris</i>	JX564871	-
<i>Alytes obstetricans</i>	AY585337	-
<i>Bombina orientalis</i>	AY585338	-
<i>Buergeria buergeri</i>	AB127977	-
<i>Bufo japonicus</i>	AB303363	-
<i>Duttaphrynus melanostictus</i>	AY458592	-
<i>Cophyla phyllodactyla</i>	EU341112	-
<i>Crinia signifera</i>	JX564860	-
<i>Discoglossus galganoi</i>	AY585339	-
<i>Euphlyctis hexadactylus</i>	NC014584	-
<i>Fejervarya cancrivora</i>	NC012647	-
<i>Heleophryne purcelli</i>	JX564867	-
<i>Hoplobatrachus tigerinus</i>	NC014581	-
<i>Hyla chinensis</i>	AY458593	-
<i>Indirana leithii</i>	KF590637, KF590627	-
<i>Kaloula pulchra</i>	AY458595	-
<i>Limnodynastes salmini</i>	JX564877	-
<i>Mantella madagascariensis</i>	AB212225	-
<i>Microhyla ornata</i>	DQ512876	-
<i>Nasikabatrachus sahyadrensis</i>	JX573176, JX573185	12S: AY364360, AY425726, JX573185; 16S: GU136108, AY364381, AY425725, JX573176
<i>Nasikabatrachus bhupathi</i> sp. nov.	MF182111, MF182110	12S: MF182111 16S: MF182110
<i>Pelobates cultripes</i>	AJ871086	-
<i>Phrynomantis microps</i>	JX564886	-
<i>Pipa carvalhoi</i>	NC015617	-
<i>Polypedates megacephalus</i>	AY458598	-
<i>Quasipaa spinosa</i>	FJ432700	-
<i>Rana plancyi</i>	EF196679	-
<i>Rhinophrynus dorsalis</i>	NC021849	-
<i>Rhombophryne testudo</i>	EU341110	-
<i>Scaphiophryne madagascariensis</i>	JX564893	-
<i>Sooglossus sechellensis</i>	JF703232	JF703232
<i>Sooglossus thomasseti</i>	NC021850	NC021850
<i>Sechellophryne gardineri</i>	-	16S: DQ872919
<i>Sechellophryne pipilodryas</i>	-	16S: DQ872918
<i>Xenopus tropicalis</i>	AY789013	-
Outgroup		
<i>Leiopelma archeyi</i>	NC014691	-

Table 3. K2P genetic distances between *Nasikabatrachus bhupathi*, *N. sahyadrensis* and some sooglossids.

Taxon		1	2	3	4	5	6	7	8	9
<i>Nasikabatrachus bhupathi</i> sp.nov. (Holotype, Eastern slopes)	1	0.0 *								
		0.0 #	0.058	0.067	0.056	na	0.242	0.240	na	na
<i>N. sahyadrensis</i> (Holotype, Kattappana)	2	0.035		0.000	0.000	na	0.242	0.228	na	na
<i>N. sahyadrensis</i> (Sankaran kudi, Anaimalais)	3	0.035	0.000		0.010	na	0.245	0.232	na	na
<i>N. sahyadrensis</i> (Karian shola, Anaimalais)	4	0.035	0.000	0.000		na	0.245	0.232	na	na
<i>N. sahyadrensis</i> (Western Ghats)	5	0.035	0.000	0.000	0.000		na	na	na	na
<i>Sooglossus thomasseti</i>	6	0.215	0.214	0.214	0.214	0.214		0.055	na	na
<i>Sooglossus sechellensis</i>	7	0.203	0.205	0.205	0.205	0.205	0.058		na	na
<i>Sechellophryne pipilodryas</i>	8	0.227	0.222	0.222	0.222	0.222	0.150	0.170		na
<i>Sechellophryne gardineri</i>	9	0.238	0.227	0.227	0.227	0.227	0.149	0.155	0.065	

RESULTS

Molecular characterization of partial 12S and 16S rRNA gene and subsequent phylogenetic analysis indicated that the newly discovered population of *Nasikabatrachus* sp. in the eastern slopes of the Western Ghats significantly differed from the previously described species *N. sahyadrensis*. Further, morphological examination and acoustic characterization showed results concordant with the genetic analysis. Thus, we convincingly describe the newly discovered population from the eastern slopes as a new species.

SYSTEMATICS

Nasikabatrachus bhupathi sp. nov. (Figures 1, 3, 4b, 5b, 6c–d, 7b,d, 10; Tables 1, 4)

Holotype

Adult male ZSIA 14153 (fig. 3), collected by S. J. Janani from a stream flowing through a farm outside the Watrap Range of the Srivilliputhur Grizzled Giant Squirrel Wildlife Sanctuary, at 200 m a.s.l. (fig. 2). The specimen was calling from a burrow entrance, about 5 m from the seasonal second order stream, immediately after rain, at around 17:30 h, on 9 October 2012.

Paratypes

Adult male ZSIA 14174 collected by S. J. Janani, at around 20:30 h, on 17 October 2012, at the same locality as the holotype; this specimen was calling at the time of collection, immediately after rain.

Other paratypes: four imagos (ZSIA 14154–7) and six tadpoles at Gosner (1960) stages 29–44 (ZSIA 14158–63), collected from a stream at the same locality as the holotype, by S. J. Janani, during the 2011 breeding season. These imagos were raised from tadpoles at advanced stages and preserved (fig. 10).

Etymology

The species epithet commemorates Dr. S. Bhupathy, a noted scientist and a field herpetologist, who passed away due to an ill-fated accident while conducting herpetological surveys in Agasthyamalai, Western Ghats on April 28, 2014.

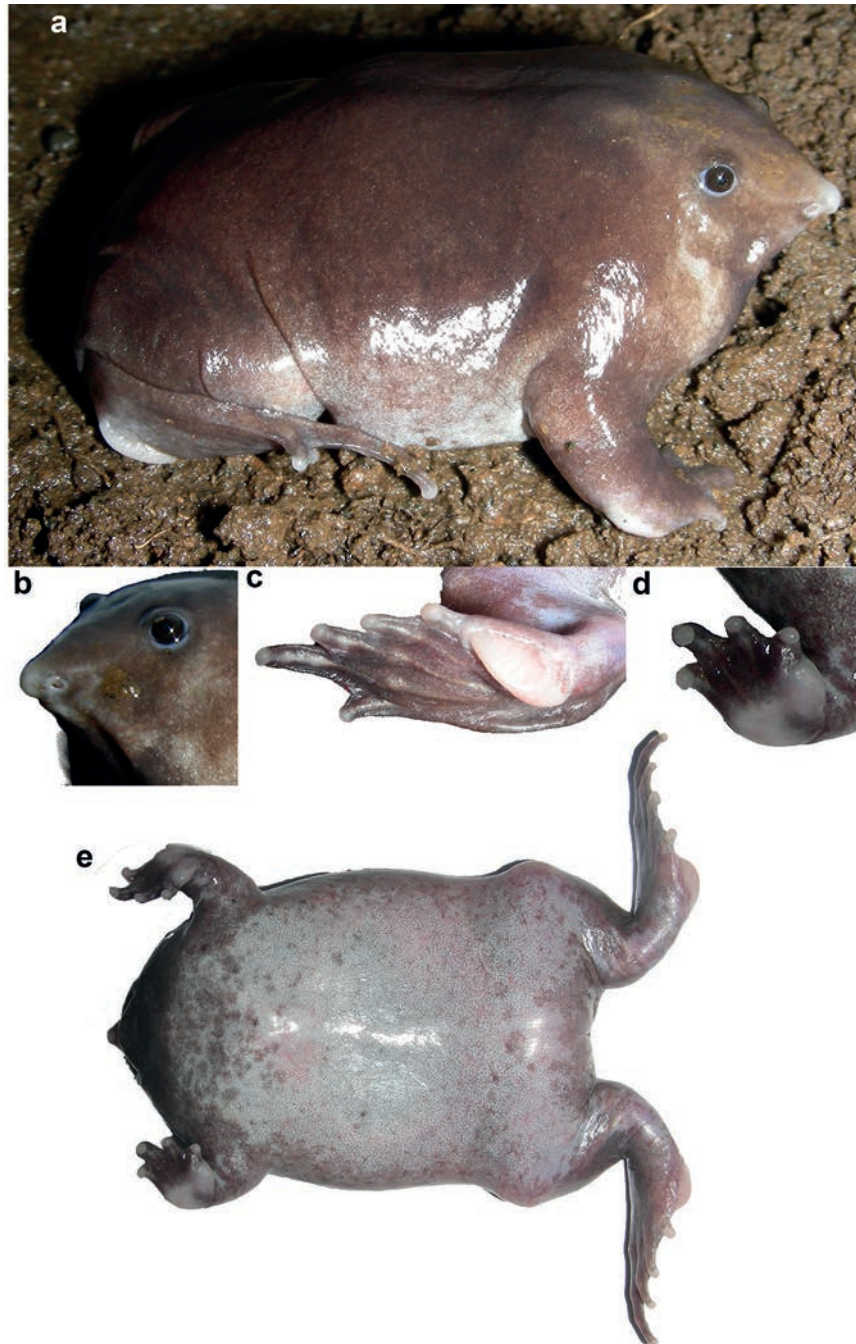


Figure 3. Holotype of *Nasikabatrachus bhupathi*. (a) dorsolateral view; (b) anterior view of head showing fleshy protuberance on the snout; (c) underside of foot, showing hypertrophied shovel-shaped inner metatarsal tubercle; (d) underside of hand showing palmar tubercles; (e) ventral view.

Diagnosis

The new species is morphologically assigned to the genus *Nasikabatrachus* on the basis of the following three external character states of adults: (1) elongated fleshy protuberance on the snout, hardened at the tip (fig. 4); (2) distinct skin indent running below eye to nostril, with another skin indent running from below posterior corner of eye to below mouth; (3) extremely hypertrophied prehallux comprising four ossified distal elements, curling up dorsally and extending over the inner toe. In addition, this taxon has small eyes with round pupils and a distinctive full blue ring of tissue around the eye (visible in live animals), an acutely pointed snout, nostril openings directed posteriorly, a globular compact body, tympanum not externally visible, and a lack of both the anterior and posterior palatal folds, a combination of characters not occurring together in any other known genus of frogs than *Nasikabatrachus*.

Nasikabatrachus bhupathi is diagnosed from the only known congener, *N. sahyadrensis*, on the basis of the following osteological characters: (1) the dorsal crest of the urostyle extends to just about half of urostyle length and is sharply tapered posteriorly in *N. sahyadrensis* (fig. 5a), whereas it is longer, more strongly developed, and more gently tapering posteriorly in *N. bhupathi* (fig. 5b); (2) transverse processes of PSV8 are shorter and acutely anterolaterally orientated in *N. sahyadrensis* (fig. 6a–b), but are longer and only slightly anterolaterally orientated in *N. bhupathi* (fig. 6c–d); (3) the transverse processes on PSV4 are approximately equal in length in *N. sahyadrensis* (fig. 6a–b) but are slightly shorter than those of PSV3 in *N. bhupathi* (fig. 6c–d); (4) the neural spines on PSV4–8 of *N. sahyadrensis* (fig. 6a) are less well-developed and flatter than those in *N. bhupathi* (fig. 6c), where neural spines are better developed and extended into a semi-cylindrical posterior projecting spine on the anterior half of each vertebra; (5) the transverse processes of PSV1+2 are much broader in *N. sahyadrensis* (fig. 6a) and bear a well-developed, near-isosceles triangle-shaped accessory process covering most of the anterior edge of the transverse process body, whereas the transverse processes of PSV1+2 are much narrower in *N. bhupathi* (fig. 6c) and the accessory process on the transverse processes of PSV1+2 are weakly developed; (6) the transverse processes of PSV3 have a distinct accessory process in *N. sahyadrensis*, visible in ventral view in fig. 6b, but this is not evident in *N. bhupathi* (fig. 6d). In external morphology, the two species differ in coloration. *Nasikabatrachus sahyadrensis* has a purple-brown coloration of dorsum, whereas *N. bhupathi* lacks the purple tinge on the dorsal

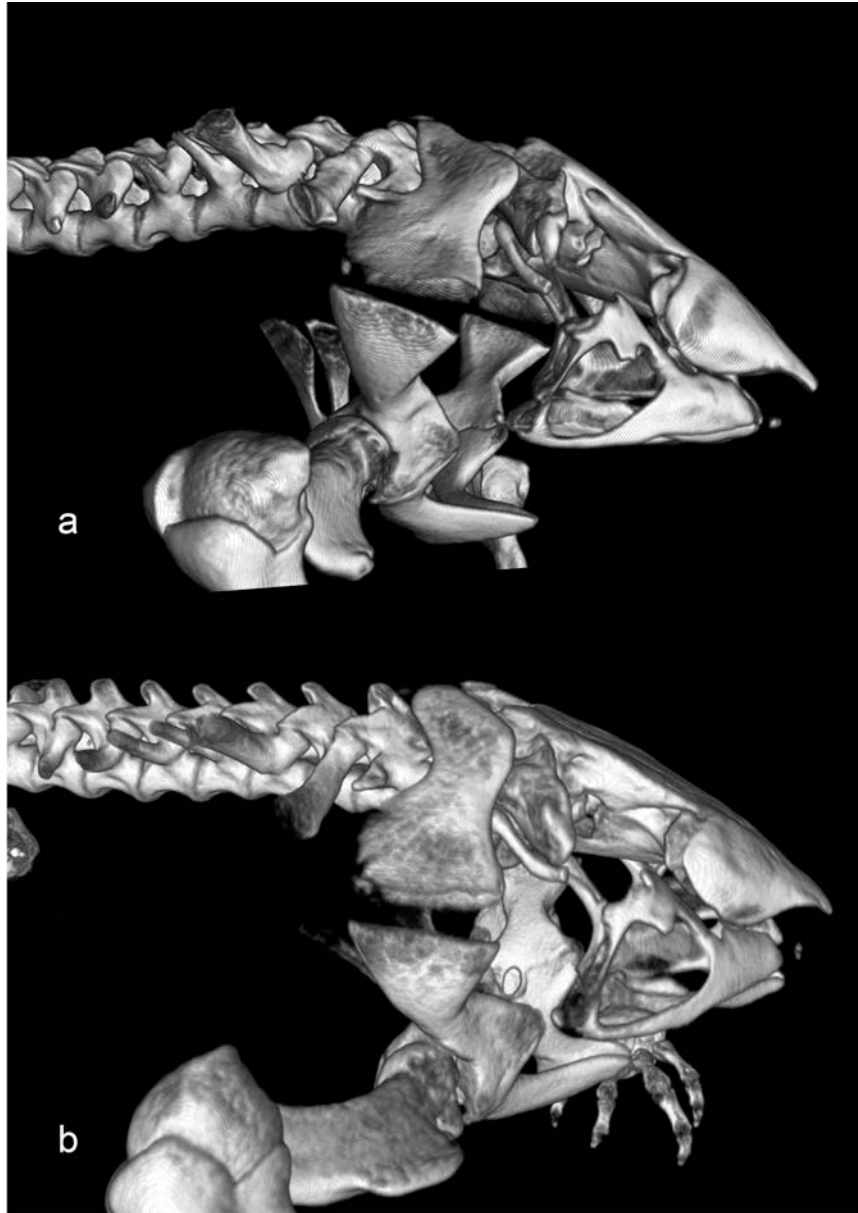


Figure 4. Skulls and anterior vertebrae of *Nasikabatrachus*, in lateral view. (a) *N. sahyadrensis* (KAUNHM 2011131); (b) *N. bhupathi* (ZSIA 14153, male), showing skull morphologies.



Figure 5. Pelvic girdles of *Nasikabatrachus*, in lateral view; (a) *N. sahyadrensis* (KAUNHM 2011131); (b) *N. bhupathi* (ZSIA 14153), showing increased development of the dorsal ridge (crista dorsalis) of the urostyle.

surfaces: the dorsal coloration of the head is light brown while the dorsum is dark brown (fig. 3). Additionally, the imagos of *N. bhupathi* also show a distinctive dark and light brown marbling with infuscation in coloration of the dorsum (fig. 10), which may be inflected with reds. Although our sample size is low, it appears that *N. bhupathi* is smaller in overall size than *N. sahyadrensis*.

Description of the holotype

A medium-size male (48.5 mm SVL, Tab. 1); abdominal skin smooth, greyish-white with faint marbling in coloration; skin on limbs smooth; skin on dorsum smooth, thick, and dark brown from vent to shoulder; head lighter brown; no dorso-lateral or transverse skin folds; body globular; head not externally distinct from body, the snout acutely pointed with a lighter coloured fleshy protuberance and a hard knob-like projection at the tip; mouth small, subterminal, ventral, and posterior to snout tip; mouth not extending posteriorly (beyond a vertical line drawn downwards from the anterior corner of the eye); a distinct indent running below the eye to the nostril, with another deeper skin fold extending from below the posterior corner of the eye to behind and below the articulation of the jaw; nostrils directed postero-ventrally, nostril positioned below the dorsal margin of the eye and located anteriorly, placed much nearer to the tip of the snout than to the eye; eyes small, pupils round, sclera blue forming a ring, in life; eye diameter much smaller than the distance between the anterior edge of the eye and the nostril; interorbital distance more than three times the width of the upper eyelid; tympanum not visible externally; vomerine teeth absent; mandibular teeth and odontoids absent; tongue small with entire rounded tip, basally attached, fluted and elongated in shape, longer than wide, lacking a medial lingual process.

Forelimbs short and muscular with a restricted range of movement; fingers unwebbed; tips rounded without subarticular, subdigital or supernumerary tubercles; palm fleshy, background color brown; tips of fingers, palmar tubercles pale white, relative length of the fingers: $3 > 2 > 1 = 4$; tips of toes rounded, rudimentary webbing between toes; outer two metatarsals forming part of the fleshy tubercle (*sensu* Poynton 1963), separated distally; no digital scutes; large, hypertrophied shovel-shaped inner metatarsal tubercle with pale white callused margin; outer metatarsal tubercle present; slight short flange on outer surface of foot; tarsal fold absent, nuptial pads absent, macroglands not evident; gular gland absent; femoral glands absent; cloacal opening directed postero-ventrally.

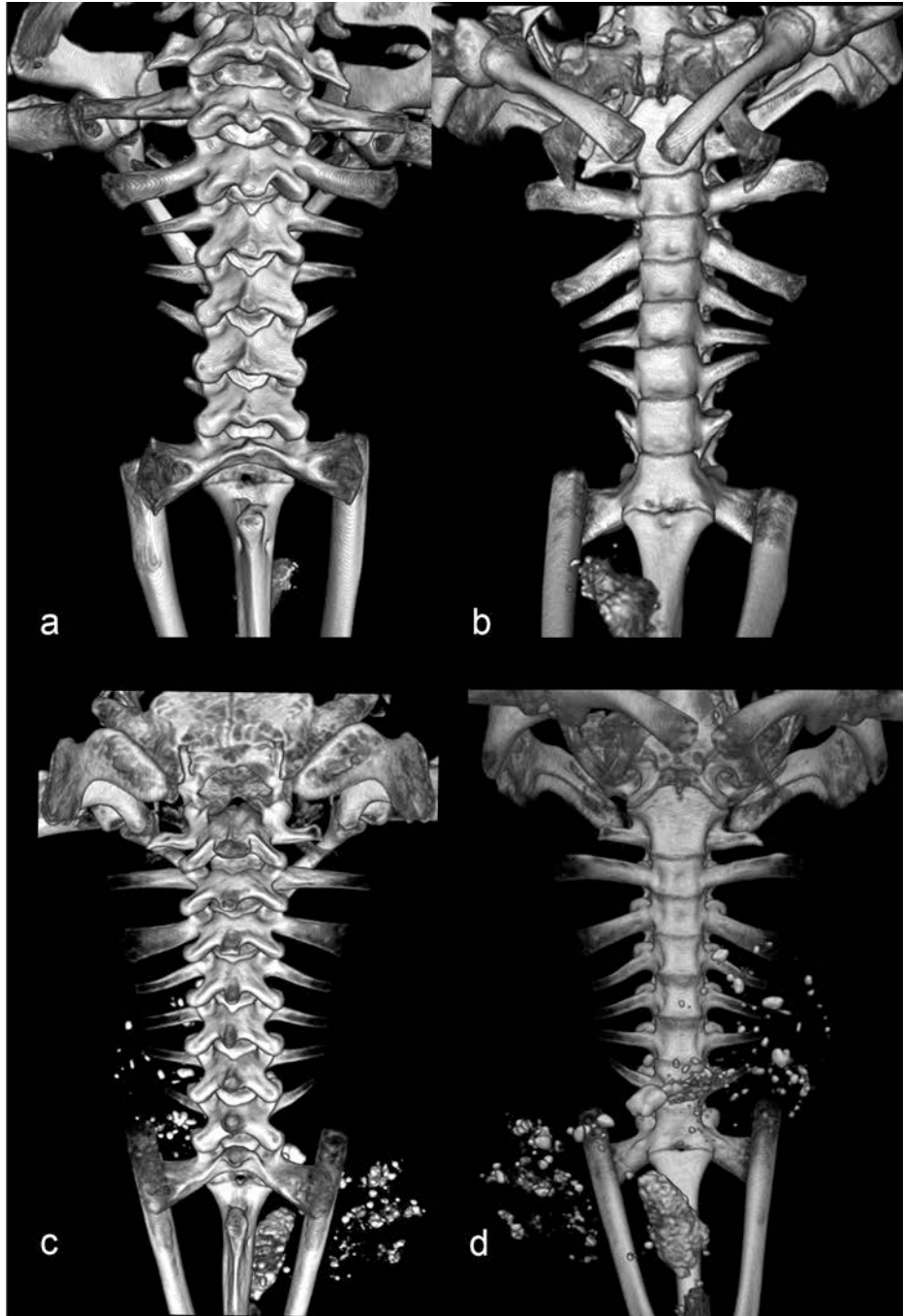


Figure 6. Vertebral columns of *Nasikabatrachus sahyadrensis* (top row, KAUNHM 2011131): (a) dorsal view; (b) ventral view; and *Nasikabatrachus bhupathi* (bottom row, ZSIA 14153): (c) dorsal view; (d) ventral view, showing longer and more acutely angled transverse processes of PSV 8 in *N. bhupathi* and thicker transverse processes with thicker anteriorly directed accessory process in *N. sahyadrensis*.

Variation

The SVL in the two adult males ranged from 45.9 to 48.5 mm.

Natural history and distribution

The currently known distribution of *Nasikabatrachus bhupathi* is restricted to three highly seasonal second order streams. The type locality is on the leeward side of the Western Ghats, which receives less rainfall than the western slopes of these mountains during southwest monsoon. Rainfall is sporadic on the eastern slopes of the Western Ghats (in 2010–2012, the average rainfall was ca. 700 mm annually). Streams on the hill slopes where *N.*

bhupathi occurs are ephemeral, with water flowing for only 3–4 months during the year (October to January). Only a few sites along the first and second order streams have stagnant water pools throughout the year. The riparian zone and the streams where the tadpoles were found have open canopy and the stream bed shows rocks of varying sizes. Other anurans found along these streams were *Duttaphrynus melanostictus*, *Fejervarya* sp., *Indirana brachytarsus* and *Sphaerotheca breviceps*. The onset of breeding calls was highly synchronous to the monsoonal showers, wherein males call only during the rain showers and for a few minutes thereafter. The frequency of calling periods increases as the frequency of intermittent rain showers increase. We did not observe a pattern of constant evening calls, as noted for *N. sahyadrensis* during the breeding season (Raj *et al.* 2011), which may be due to the differences in the rainfall patterns between the southwest and the northeast monsoons. The northeast monsoon occurs mainly due to the cyclonic depressions set in the Bay of Bengal. During this period, the eastern slopes also receive thunder showers, typical of convectional rainfall, late in the afternoon, with intense heat during the day time. In contrast to the southwest monsoon, there are very few days that are completely overcast. Therefore, calls were not heard on all days. During the observations, calls started as the rainfall commenced, and the intensity of calling declined as rain stopped. Calls were recorded close to the ground, from just the entrance (roughly 5–8 cm deep) of the burrows which were scattered within less than 5 m from the stream along the stream banks. We sometimes observed the male's vocal sacs when a torch was shone inside the burrow. Generally, the mating chorus started by one or few individuals, increase in intensity, as many other breeding males join the chorus.

Acoustic characterization

The male advertisement call of both species of *Nasikabatrachus* showed marked differences (fig. 7). The advertisement call of *N. bhupathi* consisted of 25–27 notes per minute whereas that of *N. sahyadrensis* consisted of 21–24 notes per minute. In the case of *N. bhupathi*, each note was 0.28–0.466 s in duration (mean = 0.345 ± 0.04 s; $N = 54$); and the time interval between two notes varied from 1.056 to 3.154 s, with a mean of 1.714 ± 0.44 s, $N = 43$. The frequency ranged from 1.2 to 1.8 kHz. With intensifying rainfall, calling became more intense and frequent. In *N. sahyadrensis*, each note was 0.211–0.417 s in duration with a mean of 0.377 ± 0.04 s, $N = 49$, the distance between the notes varied from 1.55–3.44 s with a mean of 2.39 ± 0.48 s, $N = 42$, and the frequency ranged from 0.6 to 1.4 kHz. The main difference in the call pattern was as follows: in *N. bhupathi* each note consisted of four pulses; whereas in *N. sahyadrensis* each note comprised three pulses, with a distinct pause, of approximately 0.1 s between the second and the third pulse.

Molecular differentiation

The preliminary BLAST results of the amplified partial mitochondrial domains (12S: 824 bp, 16S: 1330 bp) confirmed that our specimens belong to *Nasikabatrachus*. The K2P genetic distances at the individual domains (partial 12S, 16S rRNA gene) between *N. bhupathi* and all other species of *SOOGLOSSOIDEA* are presented in Table 3. There was no intra-specific variation among the holotype and the paratypes of *N. bhupathi*. The K2P genetic distance at the partial 16S rRNA gene domain (3' end of 16S, ~ 590 bp), between *N. bhupathi* and *N. sahyadrensis* (from the western slopes) was 3.5 %. This value was greater than the cut-off value of 3 percent for species level delimitation based on genetic distances, proposed by Fouquet *et al.* (2007b), indicating the species to be candidate new species. Similarly, the K2P genetic distance at the partial 12S rRNA gene domain (370 bp), between *N. bhupathi* and *N. sahyadrensis* from western slopes ranged from 5.6 to 6.7 %. However, the K2p genetic distance at a longer fragment of 16S (1330 bp) and 12S (824 bp) rRNA gene between *N. bhupathi* and *N. sahyadrensis* was 6.0 % and 5.9 % respectively. These values indicate high level of genetic differentiation between the species.

Molecular phylogeny

All neobatrachian anurans considered in this analysis grouped into three primary subclades: *RANOIDEA*, *HYLOIDEA* and a subclade containing *HELEOPHRYNIDAE*, *MYOBATRACHIDAE* and (*NASIKABATRACHIDAE* + *SOOGLOSSOIDEA*) (fig. 8). As expected, addition of another species to *Nasikabatrachus* did not affect its position within *SOOGLOSSOIDEA*. The node that recovered *SOOGLOSSOIDEA* as sister-taxa to *HELEOPHRYNIDAE* and *MYOBATRACHIDAE* was well supported by Bayesian inference, and moderately supported by ML analysis. A second phylogenetic analysis was performed to include all the available sequences of *N. sahyadrensis*, from different localities in Western slopes of the Western Ghats (as shown in map, fig. 1), along with other sooglossids and *Heleophryne purcelli* as outgroup. The obtained tree resulted differentiating all the individuals of *N. sahyadrensis* from the western slopes as one group and *N. bhupathi* as the other (fig. 9), concordant with the results based on genetic distances.

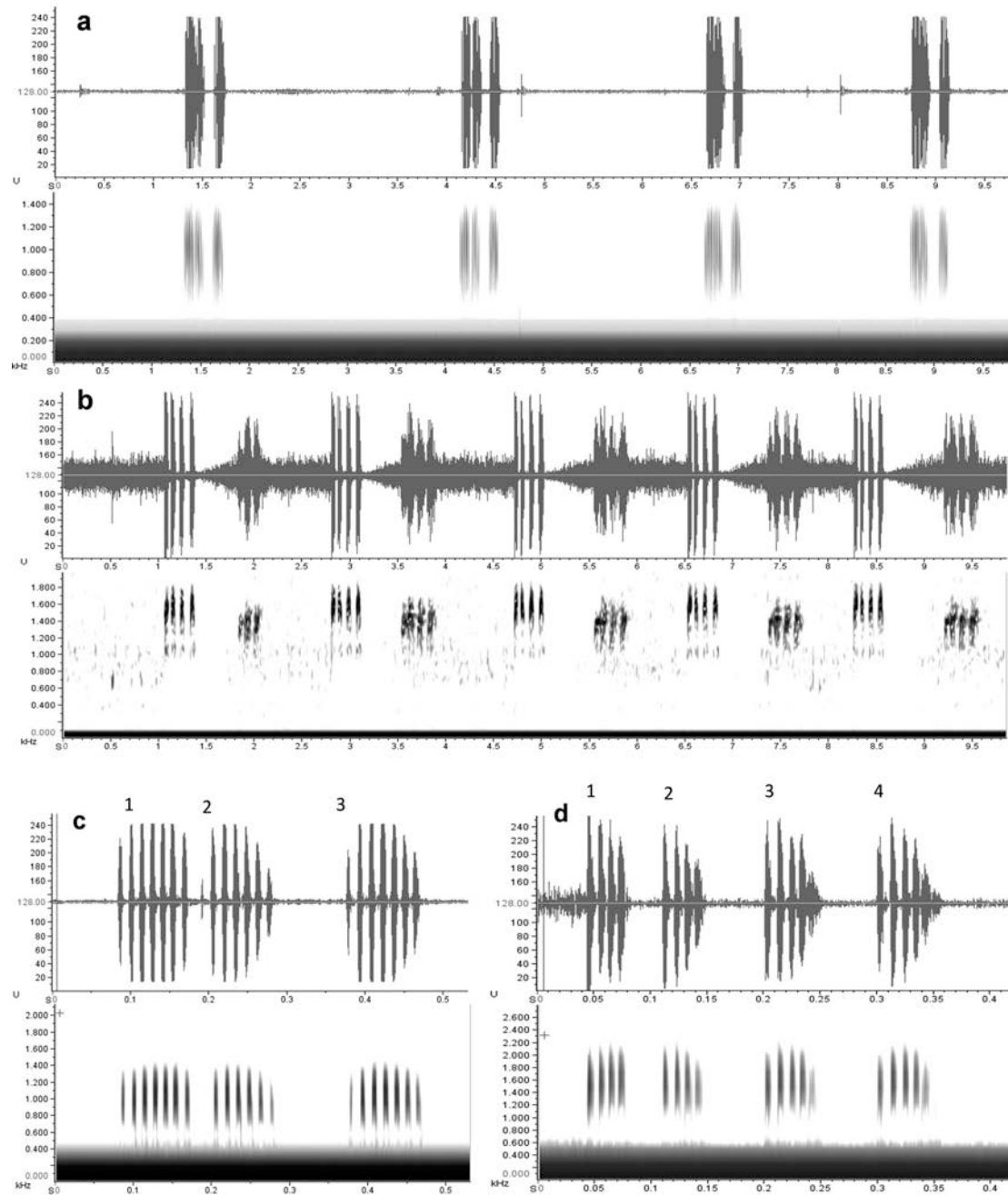


Figure 7. Advertisement calls of *Nasikabatrachus*, with waveform (above) and sonogram (below). (a) *N. sahyadrensis*, from Macaulay Natural Sounds library Catalogue No. 163897; (b) *N. bhupathi*, recorded from the type locality, with second individual calling in the background; (c) one note of call of *N. sahyadrensis* with three pulses; (d) one note of *N. bhupathi* with four pulses.

Description of the larval characteristics

Observations on the breeding behavior of *Nasikabatrachus bhupathi* were made over three seasons and found to be highly monsoon dependent. The peak of monsoon (October to November 2011) resulted in explosive breeding of this species, as visualized from the tadpole abundance. The tadpoles of *N. bhupathi* outnumbered tadpoles of other species in the streams, which is typical of explosive breeders. However, amplexus was not observed and hence egg clutch characteristics could not be inferred. There were no discernable differences in the external larval morphology between *N. bhupathi* and *N. sahyadrensis* (see the data presented in Raj *et al.* 2012 and Zachariah *et al.* 2012). The tadpoles have large ventral suctorial oral disk (a rheophilic adaptation) and a funnel-shaped vent tube opening medially. We observed no change in pigmentation on the skin until stages 25–26, when the color of the dorsum varied from medium to dark brown and the ventrum was pale whitish. The labial

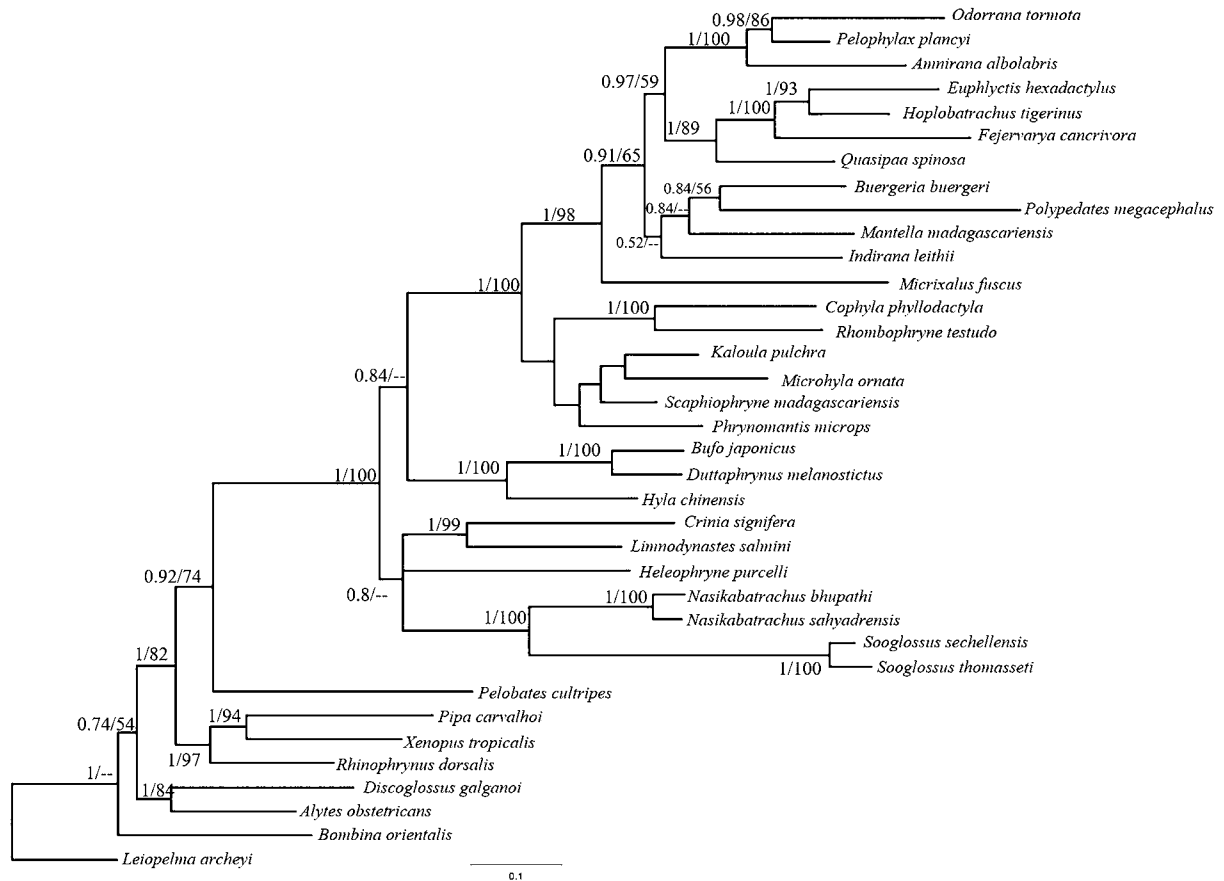


Figure 8. Phylogenetic tree inferred from concatenated two partial mitochondrial genes (12S rRNA and 16S rRNA). Presented is here a Bayesian inference tree with support values of the nodes written above each node: Bayesian posterior probabilities, followed by ML bootstrap values.

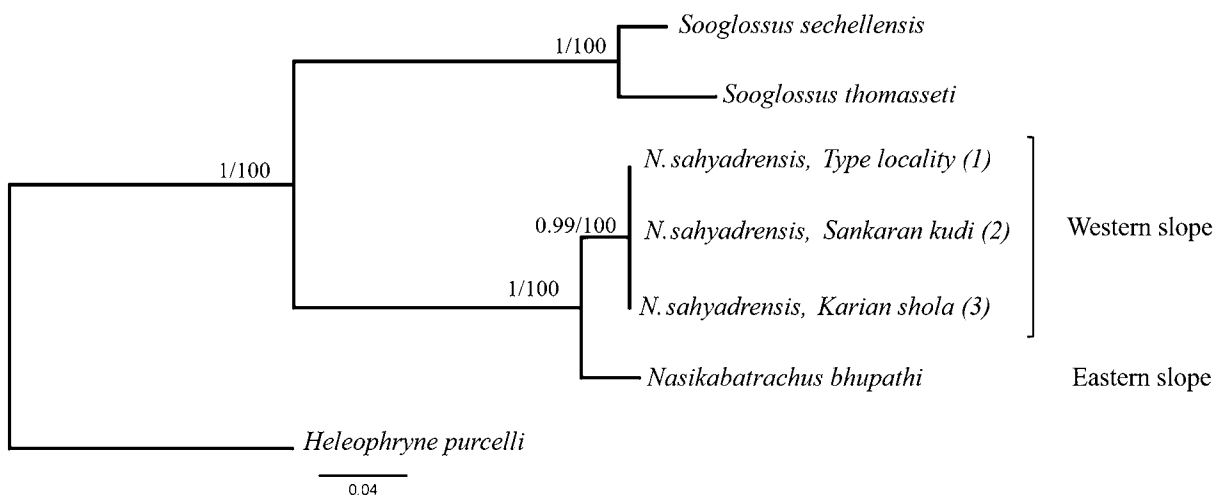


Figure 9. Phylogenetic tree based on reduced number of taxa (representing only *SOOGLOSSOIDEA*), inferred from concatenated partial 12S and 16S rRNA genes; presented is a Bayesian inference tree with support values written above each node: Bayesian posterior probabilities, followed by ML bootstrap values. 1, 2, 3 represent the locations given in fig. 1, which represents populations from the western slope of the Western Ghats.



Figure 10. External morphology of tadpoles of *Nasikabatrachus bhupathi*. (a) dorsal view; (b) ventral view at stage 36, with developing hind limbs, vent tube still present; (c) dorsal view at stage 41, with completely developed hind limbs and visible forelimb bud; (d) dorsal view at stage 42, with both forelimbs erupted; (e) ventral view at stage 42, where one of the forelimbs had erupted completely and the other is yet to erupt, absence of vent tube; (f) dorsal view at stage 43, with transforming snout; (g–i) stage 44–45, showing ventral and dorsal side, transforming oral disc and tail stub; (j) developed imago showing pigmentation pattern of brown and black mottles.

tooth row formula (LTRF) is 2/3(1). Images of various larval stages are presented in fig. 10 and the larval measurements in Table 4. The spiracle remains visible until stage 37, after which the ventral skin becomes opaque and pale white. The re-absorption of the tail occurs from stage 41 until metamorphosis is complete. The oral disc narrowed in width to a minimum in stage 45, when the specimens still had tail vestiges. The completely metamorphosed individuals showed pigmentation pattern of brown and black mottles. Developmental asynchrony with respect to disappearance of oral disc and complete re-absorption of the tail was observed in *N. bhupathi* like described in *N. sahyadrensis* by Senevirathne *et al.* (2016).

Table 4. Morphometrics of tadpoles of *Nasikabatrachus bhupathi*.

Measurements	<i>Nasikabatrachus bhupathi</i> sp. nov.					
	Paratypes (Tadpoles)					
	ZSIA 14162 Stage 29 (n = 1)	ZSIA 14163 Stage 30 (n = 1)	ZSIA 14159 Stage 38 (n = 1)	ZSIA 14158 Stage 39 (n = 1)	ZSIA 14161 Stage 40 (n = 1)	ZSIA 14160 Stage 44 (n = 1)
IND	1.6	1.7	2.4	2.2	2.0	2.0
IOD	4.3	4.3	5.5	5.2	6.3	5.1
BL	15.5	14.8	19.9	21.7	17.6	20
TL	19.3	25.8	38.7	28.9	33.6	2.7
TH	5.7	4.7	5.9	6.1	8.2	1.4
TW	3.5	3.8	4.9	4.8	5.7	2.7
ESD	7.7	8.6	11.4	11.1	10.2	6.2
NSD	5.3	5.6	7.6	7.4	6.4	3.7
BW	5.8	6.1	10.0	10.6	10.5	10.4
BH	5.1	5.4	7.4	7.8	8.0	6.0
ODD	7.4	7.6	10.4	8.2	10.2	6.5

DISCUSSION

Cryptic species are a window to biodiversity that can inform on phylogeography and help for the designation of conservation units (Bickford *et al.* 2007; Pfenninger & Schwenk 2007; Trontelj & Fišer 2009); Among recent studies, amphibians show the largest number of cryptic species identified through molecular data, across different biomes, particularly in the tropics where they show high species richness (Wynn & Heyer 2001; Camargo *et al.* 2006; Stuart *et al.* 2006; Elmer *et al.* 2007; Fouquet *et al.* 2007a–b; Vieites *et al.* 2009; Funk *et al.* 2012). Between cryptic sister-species, acoustic signals might be much more differentiated than external morphological characters due to higher selection pressure in species recognition during reproduction (Funk *et al.* 2012). Many identifications of cryptic speciation have been recorded among the widespread “species”, prompting Stuart *et al.* (2006) to suggest that there may not be any single widespread species of forest-dwelling anuran and that cryptic lineages occurring in sympatry might be the rule rather than the exception in Southeast Asia. Here, we describe one such cryptic speciation event within *Nasikabatrachus*, using distinct acoustic, skeletal and molecular data, all of which validate the new species.

The evolution and diversification of the two species of *Nasikabatrachus*, both endemic to the Western Ghats, is an example of external morphological stasis. Both taxa are obligate hypogean species and morphological stasis is generally observed in species occupying caves and subterranean biomes (Porter 2007; Juan *et al.* 2010). *Nasikabatrachus* is highly adapted for fossoriality (Senevirathne *et al.* 2016). Regarding the relative role of dispersal and colonization *versus* vicariance events influencing the speciation of this lineage, we consider vicariance to be much more plausible for this subterranean specialist, which is not expected to be highly vagile. Further, the rheophilic specialization of the tadpole to river cascades, and the reproduction timed co-incidentally with disparate monsoon seasons, could further facilitate vicariance. The distribution is expected to be confined to moist soil near the streams, or with significant groundwater. *Nasikabatrachus* lives and feeds underground, therefore hard, dry soil and rock (from mountain uplift) is expected to present an insurmountable barrier to burrowing and feeding, preventing this species from dispersing far.

Our knowledge about the distribution pattern of *Nasikabatrachus bhupathi* is currently limited; further surveys and detailed studies on the geographic range and population size in the Western Ghats are required. We expect the distribution to be narrow and likely confined to the foothills of the eastern slopes of the Western Ghats,

roughly between 8° and 10°N. However, it is evident that the population of *N. bhupathi* at its type locality needs to be protected. Within the Watrap Range, one of the three second-order streams (where breeding populations were observed) is disturbed and polluted, as it falls in a pilgrimage route. Measures need to be taken to stop degradation and restore the stream habitat for this unique and rare species of frog.

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