



SCALING NEW HEIGHTS: FIRST RECORD OF BOULENGER'S LAZY TOAD *SCUTIGER BOULENGERI* (AMPHIBIA: ANURA: MEGOPHRYIDAE) FROM HIGH ALTITUDE LAKE IN SIKKIM HIMALAYA, INDIA

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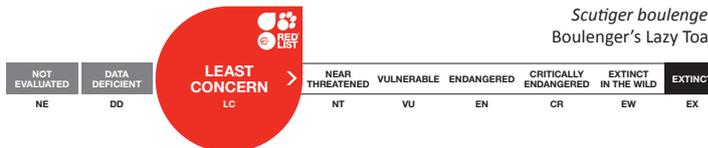
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Abstract: Frogs in the genus *Scutigera* of the family Megophryidae are the highest altitude frogs in the world, and are endemic to eastern Himalaya. Here, we report the occurrence of *Scutigera boulengeri* (Bedriaga, 1898) in one of the highest altitude lakes in the world, Lake Gurudongmar. This is the first report of *Scutigera boulengeri* from India and the first record of any amphibian species to be found at an altitude as high as 5270m, thus setting a world record for the highest altitude frog. *Scutigera sikkimensis* (Blyth, 1855) is another species of the genus *Scutigera* found in this region. This species is often confused with *Scutigera boulengeri* due to similar morphology and habitat overlap. We provide a detailed account of both *S. boulengeri* and *S. sikkimensis* based on morphology and molecular identification techniques.

Keywords: Amphibian, Sikkim, glacier, lake Gurudongmar, *Scutigera boulengeri*, *Scutigera sikkimensis*.

The Himalayan region is one of the 35 biodiversity hotspots of the World (Mittermeier et al. 2004). This region has a high altitudinal range from 300–8598 m and harbors several endemic taxa. The high altitude flora and fauna of this region are important for global biological diversity because there are very few places on Earth with such a unique ecosystem (Mani 1994; Shrestha et al. 2012). In Sikkim Himalaya, 23 species of amphibians are known (Chettri et al. 2011); some species have narrow distribution range, and for many, there is still a lack of knowledge about their distribution, life history and ecology (Acharya & Vijayan 2011; Chettri et al. 2011). These areas, due to their inaccessibility, have not been surveyed extensively, especially for amphibians.



Scutigera boulengeri
Boulenger's Lazy Toad



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Among the several restricted taxa in the Himalayan region is the genus *Scutiger*, belonging to the family Megophryidae. It is one of the most diverse families of non-neobatrachian frogs. Twenty species of *Scutiger* occur in the high altitudes of the Tibetan plateau in southwestern China, Myanmar, Nepal and northern India (Frost 2015). One interesting species in this genus *Scutiger boulengeri*, commonly known as Boulenger's Lazy Toad or Xizang Alpine Toad, holds the record for being the highest altitude frog in the world (Hock 1964), found at altitudes up to 5100m. *Scutiger boulengeri* is known to occur in northwestern Nepal, and southeastern Qinghai, eastern & southern part of Xizang, southern Gansu and northwestern Sichuan provinces in western China (Ohler et al. 2014).

Another species of *Scutiger* found in Sikkim is *Scutiger sikimmensis*, commonly known as the Sikkim Snow Toad. *Scutiger boulengeri* can be easily mistaken for *S. sikimmensis*, as they are morphologically similar. *Scutiger sikimmensis* is very common in the high altitudes of Sikkim Himalaya. In this paper, using an integrative taxonomic approach, we delineate *S. boulengeri* from *S. sikimmensis* and hence confirm the presence of two species of *Scutiger* in Sikkim.

MATERIALS AND METHODS

Sampling

A total of four individuals of *S. boulengeri* and four individuals of *S. sikimmensis* collected from five locations in Sikkim were included in this study (Fig. 1). The specimens are presently deposited in ATREE museum, Bangalore, India and will be deposited in the Natural History Museum of Sikkim as per the agreement with the forest department (15/DIR(REE)/FEWMD/GOS/2011) once the museum becomes functional. Latitude and longitude were recorded using Garmin eTrex Vista H GPS with the datum set to WGS 84.

Specimens were fixed in 5% formalin for 12 to 18 hours and were subsequently transferred to 70% ethanol. Samples for molecular analysis were taken from hind limb muscle tissue, and were preserved in 70% ethanol prior to fixing in formalin.

Morphometry

Morphometric data were recorded using digital caliper (+/- 0.1mm). All measurements follow Ohler et al. (2011). The measurements were used to compute the Principle Component Analysis (PCA) between the two species. For PCA, data were transformed into their base 10 logarithm to correct for size and analysis was performed in PAST 3 (Hammer et al. 2001).

Body: SVL snout-vent length. Head: EL eye length; EN distance from anterior corner of eye to posterior edge of nostril; HL head length from posterior corner of mandible to tip of snout; HW head width, at the angle of jaws; IBE distance between posterior corners of eyes; IFE distance between anterior corners of eyes; IN internarial distance; IUE minimum distance between upper eyelids; MBE distance from posterior corner of mandible to posterior corner of eye; MFE distance from posterior corner of mandible to anterior corner of eye; MN distance from posterior corner of mandible to posterior edge of nostril; NS distance from anterior edge of nostril to tip of snout; SL distance from anterior corner of eye to tip of snout; UEW maximum width of upper eyelid. Forelimb: FLL forearm length, from elbow to base of outer palmar tubercle; HAL hand length, from base of outer palmar tubercle to tip of third finger; TFL third finger length, from articulation of proximal and intermediate phalange. Hindlimb: FFTF distance from maximum incurvation of web between fourth and fifth toe to tip of fourth toe, toes being spread; FL thigh length, from vent to knee; FOL foot length, from base of inner metatarsal tubercle to tip of fourth toe; FTL fourth toe length, from articulation of proximal and intermediate phalange; ITL inner toe length; MTFF distance from distal edge of metatarsal tubercle to maximum incurvation of web between fourth and fifth toe, toes being spread; MTTF distance from distal edge of metatarsal tubercle to maximum incurvation of web between third and fourth toe, toes being spread; TFOL length of tarsus and foot from base of tarsus to tip of fourth toe; TFTF distance from maximum incurvation of web between third and fourth toe to tip of fourth toe, toes being spread; TL shank length.

DNA extraction, amplification and sequencing

Genomic DNA extraction was carried out using thigh tissue samples preserved in 70% alcohol. The extraction was carried out following the protocol described in Vences et al. (2012). We used the primers 16Sar-L 5'-CGCCTGTTTATCAAAAACAT-3' and 16Sbr-H 5'-GAGGGTGACGGGCGGTGTGT-3' to amplify the fragment of the mitochondrial 16S ribosomal RNA gene following Hebbbar et al. (2015). The PCR cycling procedure was as follows; initial denaturation at 94°C for 3 mins, 36 cycles of 94°C for 45 sec, 55°C for 1 min and 72°C for 45 sec and a final extension step at 72°C for 10 mins. Amplified PCR products were sent for purification and sequencing to AMNION Sequencing Service, Bangalore, India.

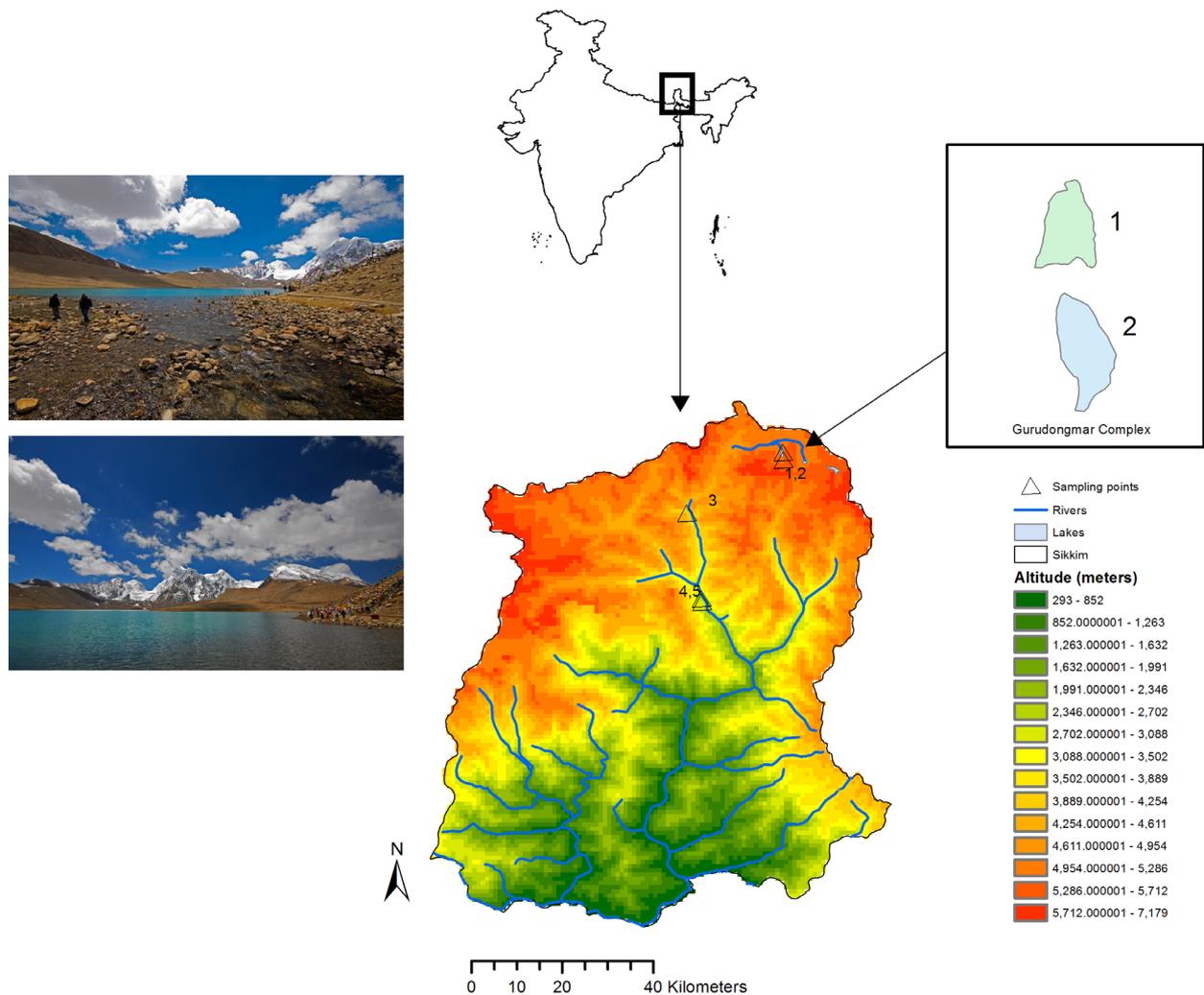


Figure 1. Sampling Locations. 1,2 - Gurudongmar Complex ($28^{\circ}01'34.79''\text{N}$ & $88^{\circ}42'51.72''\text{E}$; $28^{\circ}00'34.17''\text{N}$ & $88^{\circ}42'56.69''\text{E}$; 3 - Chopta Valley ($27^{\circ}54'10.944''\text{N}$ & $88^{\circ}31'29.6034''\text{E}$); 4,5 - Lachen ($27^{\circ}43'54.7572''\text{N}$ & $88^{\circ}33'13.428''\text{E}$; $27^{\circ}43'32.016''\text{N}$ & $88^{\circ}33'15.4116''\text{E}$).

Sequence Data Analysis

The sequence chromatogram obtained were edited using Chromas (Version 2.4, Copyright 1998–2012; Technelysium Pvt. Ltd., South Brisbane, Australia) and the BLAST (Altschul et al. 1990) analysis was performed with the sequences as queries to identify similar nucleotide sequences in the NCBI sequence database. Along with the sequences with highest homology, maximum query coverage and maximum score, the available DNA sequences of *Scutigera* species were downloaded as FASTA format from the GenBank. We obtained the sequences of *S. glandulatus* and *S. boulegeri* from the GenBank and these were included in the analyses (Appendix 1). These sequences along with the ones obtained from the current study were aligned using MUSCLE (Edgar 2004) nested in MEGA 5 (Tamura et al. 2011). All sequences were deposited in GenBank (Accession no. KR781480 –

KR781486). The best fit model of sequence evolution/ DNA substitution was determined using J-Model Test Version 2.1.7 (Darriba et al. 2012). The DNA substitution models chosen using the Akaike information criterion (AIC) in jModelTest ver. 2.1.7 was GTR+I+G for 16S rRNA dataset (AIC value = 2881.698, $-\ln L = 1396.84880$). Phylogenetic tree were constructed using Bayesian and Likelihood methods implemented in MrBayes Version 3.2 (Ronquist et al. 2011) and RaxML Version 1.3.1 (Silvestro & Michalak 2012) respectively. Ten million generations were executed in MrBayes out of which 10% (10,000 trees) were discarded as 'burn in'. The bootstrap support was determined for 1000 pseudoreplicates for the ML tree using rapid bootstrapping in RAXML Version 1.3.1. The convergence of MCMC chains was determined visually by the software TRACER Version 1.6 and by analyzing Potential Side Reduction Factor (PSRF). The

final tree was visualized using FIGTREE (Rambaut 2009).

RESULTS AND DISCUSSION

Four males and four females of the *Scutiger* species were used for both the morphometric as well as genetic analysis. Of these, two males and two females belonged to each of the two species, *S. boulengeri* and *S. sikimensis*. *Scutiger sikimensis* was collected from its type locality, Sikkim (Theobald 1868) and this is the first sequence submission of this species to GenBank. The morphometric data analysis conducted confirmed that the individuals of the genus *Scutiger* found in Sikkim are two distinct species namely *S. boulengeri* and *S. sikimensis* (Table 1; Image 1). The sequence analysis confirmed that these are two distinct species (Fig. 2). The PCA analysis based on morphometric data also supported the genetic analysis (Fig. 3). Thus, both

morphological characters as well as molecular tools clearly established that these are two distinct species of *Scutiger*.

The obtained sequences of individual species of *Scutiger* comprised of 484bp out of which there were 54 parsimony informative sites. The phylogenetic tree based on Maximum Likelihood analysis showed distinct clusters corresponding to *S. sikimensis*, *S. glandulatus* and *S. boulengeri*. The nodes with poor support (<50%) are not shown (Fig. 2). The sequences, which were attributed to *S. boulengeri* based on morphological characters, namely KR781481, KR781482 and KR781483, formed a distinct cluster with *S. boulengeri* reported from China, thus conforming the identity of the species. The pairwise genetic distance (K2P) between *S. boulengeri* from the current study and Fu et al. (2007) study were equal to or less than 3% (Table 2).

Table 1. Morphometric data of *S. boulengeri* and *S. sikimensis*. All measurements are in mm.

Characters	<i>Scutiger boulengeri</i>				<i>Scutiger sikimensis</i>			
	Male #1	Male #2	Female #1	Female #2	Male #1	Male #2	Female #1	Female #2
SVL	48.5	48.9	52.0	53.0	48.0	48.5	51.0	52.0
HW	16.3	16.6	17.8	18.0	16.5	16.9	17.7	17.9
HL	13.6	14.5	13.3	13.5	13.5	14.0	13.0	13.2
MN	11.7	11.7	11.9	11.9	12.0	12.0	12.0	12.0
MFE	8.2	9.1	9.1	9.2	8.5	8.8	8.8	8.7
MBE	4.4	4.4	4.8	4.9	4.6	5.1	5.0	5.1
IFE	7.3	7.6	7.0	7.2	7.4	7.9	7.1	7.3
IBE	13.1	13.2	13.4	13.6	12.9	13.7	13.1	13.2
IN	4.0	4.1	4.0	4.1	3.9	4.2	4.0	4.0
EN	3.3	3.3	3.4	3.4	3.0	3.0	3.0	3.0
EL	5.3	5.6	6.0	6.1	5.0	5.6	5.6	5.8
SN	2.1	2.1	2.2	2.2	2.5	2.5	2.5	2.6
SL	6.1	6.1	6.1	6.2	5.6	5.7	5.6	5.7
IUE	4.0	4.3	4.3	4.3	4.4	4.4	4.4	4.4
UEW	2.8	3.4	4.0	4.0	3.0	3.7	3.8	3.7
HAL	11.3	11.3	11.7	11.8	12.0	12.4	12.4	12.5
FLL	12.3	13.0	12.9	13.0	12.1	12.0	12.0	12.0
TFL	7.5	7.4	7.5	7.5	7.0	7.0	6.9	7.0
FL	19.1	20.4	18.6	18.7	20.7	21.6	19.7	19.8
TL	18.0	18.8	16.9	17.0	18.4	18.3	16.4	16.5
TFOL	29.5	32.8	31.5	32.2	31.8	32.0	30.0	31.0
FOL	21.2	22.5	22.6	22.6	20.0	20.0	20.0	20.0
FTL	12.0	12.0	12.3	12.3	12.0	12.0	12.0	12.0
ITL	4.5	4.5	4.3	4.5	4.1	4.0	3.9	4.0
MTTF	13.1	13.2	13.3	13.4	Rudimentary webbing			
TFTF	7.5	7.6	7.9	8.0				
MTFF	13.6	13.6	14.0	14.0				
FFTF	8.9	8.8	9.0	9.0				



Image 1. A–E - *Scutiger sikimensis*. A - Live specimen, (B–E - Preserved specimen) B - ventral view, C - dorsal view, D - ventral view of left foot, E - dorsal view of right hand; F–J - *Scutiger boulengeri*. F - live specimen, (G–J - preserved specimen) G - ventral view, H - dorsal view, I - dorsal view of left foot, J - dorsal view of right hand.

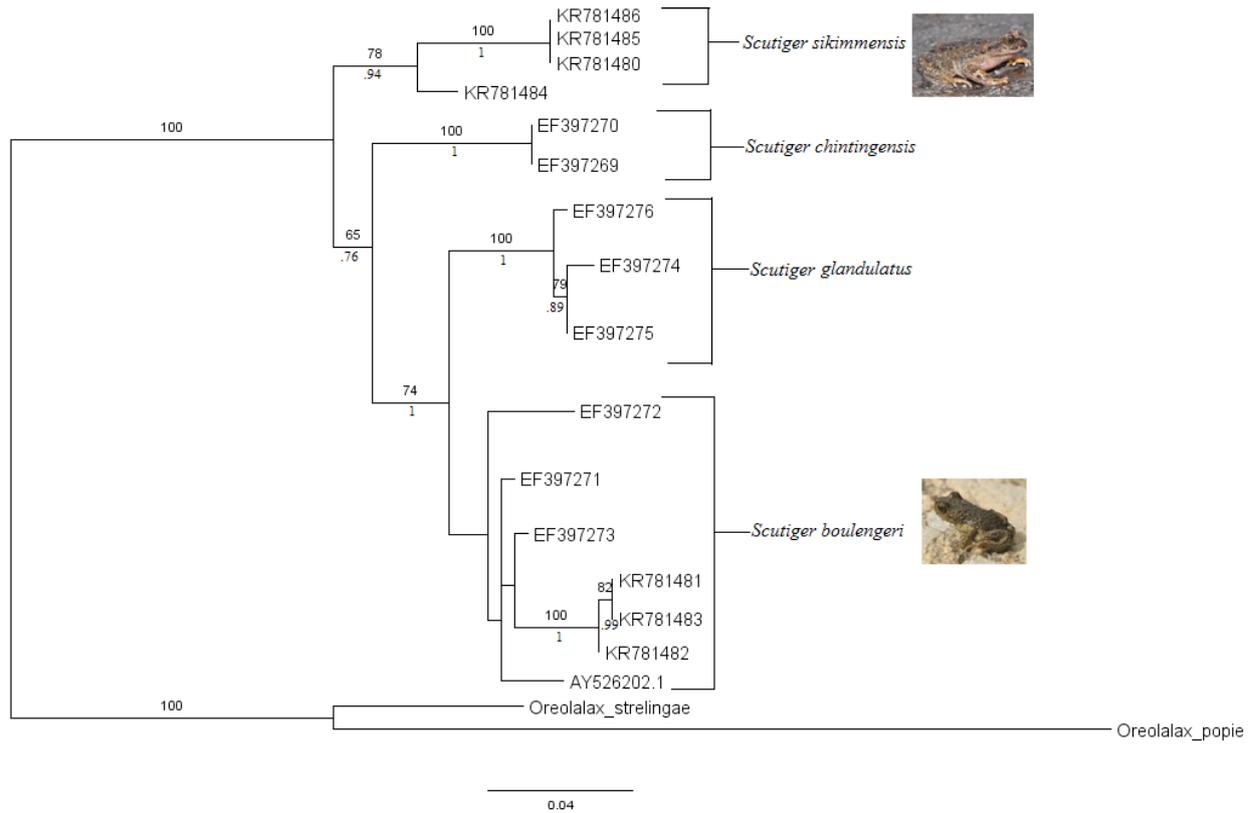


Figure 2. Molecular phylogenetic tree of *S. boulengeri*, *S. glandulatus* and *S. sikimmensis* using 16S rRNA dataset with *Oreolalax* spp. as outgroup. The values above the branches are Maximum Likelihood Bootstrap and below the branches are Bayesian Posterior Probability (BPP) generated using MrBayes. The nodes having value below 50% bootstrap and BPP 0.75 are not shown

Genus *Scutiger* is restricted to the high altitudes of the Himalaya. Here we report the occurrence of *S. boulengeri* for the first time in India, from a high altitude lake in Sikkim. This species was found in the glacier Lake Gurudongmar Complex 1 and 2 (28°01'34.79"N & 88°42'51.72"E; 28°00'34.17"N & 88°42'56.69"E) at an elevation of 5160m and 5270m respectively, which is the highest altitude ever recorded for an amphibian species.

At present, 20 species of *Scutiger* have been reported out of which 15 are endemic to China (Frost 2015). Only two species (*S. sikimmensis*, *S. nyingchiensis*) have been reported so far from India (Molur & Walker 1998). In this paper, we report *S. boulengeri* for the first time from the Indian region taking the number of amphibian species known from India to 385 (Dinesh et al. 2015). The IUCN lists *S. boulengeri* as Least Concern (Ohler et al. 2004) and its population trend as unknown. In our study area in Sikkim, both species were quite abundant.

During our field survey we found *S. sikimmensis* between 2619m to 4395m. It has been reported to occur at an altitude of 2500m and has been found upto 4600m in Sikkim (Chettri et al. 2011). We recorded *S. boulengeri* at 4005m and upto 5270m.

Table 2. K2P distance between *S. boulengeri* from Fu et al. 2007 and the current study.

Fu et al. (2007)	Current study	Percent genetic distance
EF397271	KR781481	2
EF397273	KR781481	2
EF397272	KR781481	3
EF397271	KR781482	2
EF397273	KR781482	2
EF397272	KR781482	3
EF397271	KR781483	2
EF397273	KR781483	2
EF397272	KR781483	3

Compared to other terrestrial vertebrates, amphibians are relatively more vulnerable to the effects of rapid climate change as temperature can have a pervasive effect on amphibian biology, including reproduction because of their limited mobility (Donnelly & Crump 1998). A recent study by Shrestha et al. (2012) has shown that the Himalaya are among the most vulnerable regions to future climate change. According

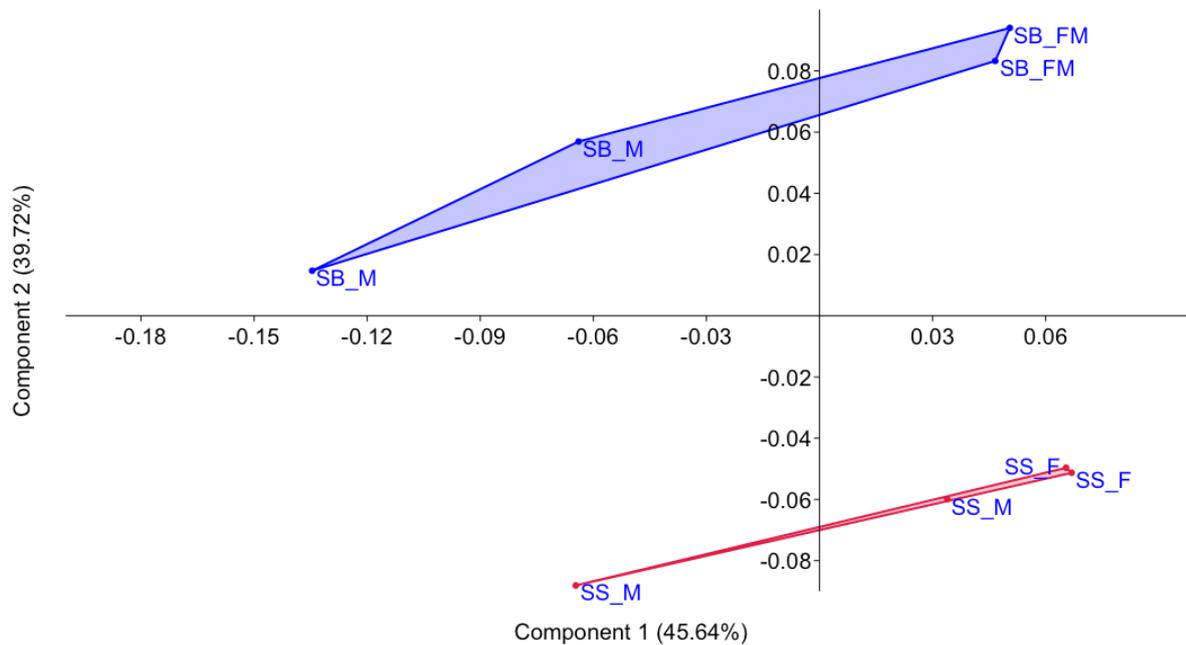


Figure 3. Principal component analysis of morphological characters for two species of *Scutiger* from Sikkim. Percent variation explained by each axis is provided in parenthesis.

(SS_M - *Scutiger sikimensis* Male; SS_F - *Scutiger sikimensis* Female; SB_M - *Scutiger boulengeri* Male; SB_F - *Scutiger boulengeri* Female)

to IPCC Himalayan glaciers are receding faster than in any other part of the world (IPCC 2007). For example, Zemu Glacier in Sikkim has retracted by 194m between 1977 and 1984 with an average retreat of 27.7m/year (IPCC 2007). Increasing mean temperature in the Himalaya has led to rapid melting of snow covers and glaciers bringing irreversible changes to the Himalayan cryosphere and rivers that are enriched by snowmelt (Bolch et al. 2012). This rapid climate change could have a serious impact on high altitude amphibians. Over 50% of amphibians are reported to be particularly susceptible to climate change (Bellard et al. 2012). Climate change also has implications on species differentiation of amphibians. A species restricted to a narrow altitudinal zone could migrate to higher altitudes due to global warming (Moritz & Agudo 2013). The future of this extremely high altitude frog remains uncertain in the face of impending climate variation and global warming. Further studies on its physiology, temperature and moisture tolerance can give an insight on a batrachian strategy to adapt to climate change.

SPECIES ACCOUNT

Scutiger boulengeri (Bedriaga, 1898) Boulenger's Lazy Toad or Xizang Alpine Toad

Materials examined (n = 4)

ATREE/Am/B03 and ATREE/Am/B08, northern Sikkim, Gurudongmar Complex 1, 28°01'34.79"N & 88°42'51.72"E, altitude 5160m; ATREE/Am/B02, northern Sikkim, Gurudongmar Complex 2, 28°00'34.17"N & 88°42'56'69"E, 5270m; ATREE/Am/B04, northern Sikkim, Chopta Valley, 27°32'42.5754"N & 88°18'47.0514"E, 3970m.

Morphological characters

SVL: Male: 45mm and 55mm; Female: 50mm and 61mm.

Head flat, wider than long. Snout rounded. Snout tip protrudes beyond lower jaw. Supratympanal fold present. Tympanum indistinct. Eyes protruding. Pupil vertical. Fingers free. Subpalmar tubercles below I and IV fingers. Toes moderately webbed. One large and oblong metatarsal tubercle present. Canthus rostralis banded. Males have nuptial spines on fingers I, II and III and a pair of small axillary and a pair of large oval pectoral shields. The belly in reproductive males is dotted with numerous small warts with black tips. Similar granules also present near cloaca. The black tip wears off in preserved

specimen if not fixed in formalin.

Coloration: Color in life olive or greenish-grey with numerous warts arranged somewhat in a longitudinal row, on the dorsal side. Ventral side yellowish in color. Dorsal pattern with slightly darker color than the body color. Dorsal pattern forms a bar between the eyes. Pupil golden.

Habit and Habitat

High altitude lakes, ponds and streams. As winter sets in towards the end of September, they come out of water and burrow into loose soil to hibernate. The hibernating individuals come out of the burrows and from under the rocks with the first rains (March/April).

Altitudinal range: 3000–5270 m.

Breeding season: May to July

Distribution

Nepal, China (Qinghai, Xizang, Gansu Sichuan). This is the first report of this species from Sikkim, India.

IUCN status: Least Concern (LC).

Scutigera sikimensis (Blyth, 1855) Sikkim Snow Toad

Materials examined (n = 4)

ATREE/Am/B05 and ATREE/Am/B09, northern Sikkim, Chopta Valley, 27°32'42.5754"N & 88°18'47.0514"E, 3970m; ATREE/Am/B06, Lachen, 27°48'42.1986"N & 88°33'7.2"E, 3273m; ATREE/Am/B07, Lachen, 27°35'16.0074"N & 88°39'4.032"E, 2575m.

Morphological characters

SVL: Male 42–62 mm; Female 45–67 mm

Head wider than long. Snout rounded, slightly protrudes over the lower jaw. Tympanum hidden. Pupil vertical. Parotid glands present. Supratympanal fold in contact with the parotoids and does not reach the upper arm insertion. Fingers free. Toe webbing rudimentary. Metatarsal tubercle absent. Males have four-rounded sub-quadrangular or oval pectoral plate-like shields, two larger mid pectoral and two smaller axillary ones. Nuptial spines present on the upper side of fingers I and II and laterally on the third finger. During reproductive phase males develop tiny warts with black tips on the sides of the head, and on the anterior side of the lower jaw, dorsal region of the legs and on the ventral region of toes IV and V.

Coloration: Dorsal color in life olive green, brown or greyish-brown with numerous warts. Dorsal pattern is

variable. Ventral region yellowish, uniform and smooth. Pupil golden.

Habit and Habitat

Found near mountain streams and rivers, grasslands, ox-bow lakes at higher elevation. They remain hidden under the stones. During breeding season males call from under the rocks at night.

Altitudinal range: 2800–4116 m.

Breeding season: June to August

Distribution

Nepal, India (West Bengal, Sikkim and Meghalaya) and China (Yadong, Cona and Nielamu).

IUCN status: Least Concern (LC).

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Appendix 1. List of *Scutigera* spp. used in phylogenetic analysis.

Species name (as indicated in the tree)	Accession No.	Locality	Reference
<i>S. boulengeri</i> 1	EF397271.1	Gansu, China	Fu et al. 2007
<i>S. boulengeri</i> 2	EF397273.1	Sichuan, China	Fu et al. 2007
<i>S. boulengeri</i> 3	EF397272.1	Sichuan, China	Fu et al. 2007
<i>S. boulengeri</i> 4	AY526202.1	Sichuan, China	Zheng et al. 2004
<i>S. boulengeri</i> 5	KR781481	Sikkim, India	Current Study
<i>S. boulengeri</i> 6	KR781483	Sikkim, India	Current Study
<i>S. boulengeri</i> 7	KR781482	Sikkim, India	Current Study
<i>S. chintingensis</i> 1	EF397270.1	Sichuan, China	Fu et al. 2007
<i>S. chintingensis</i> 2	EF397269.1	Sichuan, China	Fu et al. 2007
<i>S. glandulatus</i> 1	EF397276.1	Sichuan, China	Fu et al. 2008
<i>S. glandulatus</i> 2	EF397275.1	Sichuan, China	Fu et al. 2007
<i>S. glandulatus</i> 3	EF397274.1	Sichuan, China	Fu et al. 2007
<i>S. mammatus</i> 1	EF397279.1	Sichuan, China	Fu et al. 2007
<i>S. sikimensis</i> 1	KR781484	Sikkim, India	Current Study
<i>S. sikimensis</i> 2	KR781485	Sikkim, India	Current Study
<i>S. sikimensis</i> 3	KR781486	Sikkim, India	Current Study
<i>S. sikimensis</i> 4	KR781480	Sikkim, India	Current Study
<i>S. tuberculatus</i> 1	EF397278.1	Sichuan, China	Fu et al. 2007
<i>Oreolalax strelingae</i> 1*	KC569982.1	Vietnam	Le 2013
<i>Oreolalax popie</i> 1*	AY526210.1	Sichuan, China	Zheng 2004

* Outgroups

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