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Journal of Threatened Taxa

Building evidence for conservation globally

www.threatenedtaxa.org ISSN 0974-7907 (Online) | ISSN 0974-7893 (Print)

SHORT COMMUNICATION

OCCURRENCE OF TAMDIL LEAF-LITTER FROG *LEPTOBRACHELLA TAMDIL* (SENGUPTA ET AL., 2010) (AMPHIBIA: MEGOPHRYIDAE) FROM MANIPUR, INDIA AND ITS PHYLOGENETIC POSITION

Ht. Decemson, Vanlalsiammawii, Lal Biakzuala, Mathipi Vabeiryureilai, Fanai Malsawmdawngliana & H.T. Lalremsanga

26 May 2021 | Vol. 13 | No. 6 | Pages: 18624–18630 DOI: 10.11609/jott.7250.13.6.18624-18630



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Journal of Threatened Taxa | www.threatenedtaxa.org | 26 May 2021 | 13(6): 18624–18630 ISSN 0974-7907 (Online) | ISSN 0974-7893 (Print) https://doi.org/10.11609/jott.7250.13.6.18624-18630 #7250 | Received 05 March 2021 | Final received 13 April 2021 | Finally accepted 09 May 2021

Occurrence of Tamdil Leaf-litter Frog *Leptobrachella tamdil* (Sengupta et al., 2010) (Amphibia: Megophryidae) from Manipur, India and its phylogenetic position

SHORT COMMUNICATION

Ht. Decemson ¹, Vanlalsiammawii ², Lal Biakzuala ³, Mathipi Vabeiryureilai ⁴, Fanai Malsawmdawngliana ⁵, K. Lalremsanga ⁶

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Abstract: We present a new state record of *Leptobrachella tamdil* from Manipur State, northeastern India based on three individuals collected from Chakpi stream, Chandel District. This record represents the range extension of the species as well as the easternmost distribution record. We also provide additional morphological data as well as the first time genetic data for the species and inferred its phylogenetic position using mitochondrial 16S rRNA marker gene sequence.

Keywords: 16S rRNA, morphology, northeastern India, phylogeny, range extension.

Leptobrachid frogs are one of the most speciose groups comprising 166 species with four genera namely *Leptobrachella* Smith, 1925, *Leptobrachium* Tschudi, 1838, *Oreolalax* Myers and Leviton, 1962, and *Scutiger* Theobald, 1868. The Tamdil Leaf-litter Frog belongs to the genus *Leptobrachella* which consists of 86 congeners that are presently known from southern China, northeastern India, Myanmar through Thailand, Vietnam to Malaysia, Borneo, and Natuna Island (Frost 2021). *Leptobrachella* tamdil was originally described as *Leptolalax tamdil* based on two specimens collected from the Tamdil National Wetland, Mizoram, India (Sengupta et al. 2010). It had been known only from its type locality for about a decade. An additional specimen was reported from Dampa Tiger Reserve (DTR) (23.387–23.705N; 92.273–92.431E), Mamit District, Mizoram near the Bangladesh international boundary by Vanlalsiammawii et al. (2020). Herein, we report the occurrence of *L. tamdil* from Chakpi Stream, Chandel District, Manipur State with comments on the taxon's phylogenetic position inferred using partial sequences of mitochondrial 16S rRNA gene.

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METHODS

Herpetological surveys were carried out in two different bouts. The first trip was conducted during 16 December 2020 to 5 January 2021, and the second trip during 18 January 2021 to 24 January 2021, with a total

Editor: S.R. Ganesh, Chennai Snake Park, Chennai, India.

Date of publication: 26 May 2021 (online & print)

Citation: Decemson, Ht., Vanlalsiammawii, L. Biakzuala, M. Vabeiryureilai, F. Malsawmdawngliana & H.T. Lalremsanga (2021). Occurrence of Tamdil Leaf-litter Frog Leptobrachella tamdil (Sengupta et al., 2010) (Amphibia: Megophryidae) from Manipur, India and its phylogenetic position. Journal of Threatened Taxa 13(6): 18624–18630. https://doi.org/10.11609/jott.7250.13.6.18624-18630

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Funding: National Mission on Himalayan Studies (NMHS), Uttarakhand, Government of India (Grant No: GBPNI/NMHS – 2017/MG – 22/566); Department of Biotechnology, New Delhi, Government of India (Grant No: DBT-NER/AAB/64/2017).







Competing interests: The authors declare no competing interests.

Acknowledgements: Heartfelt gratitude to Dr. A.K. Joshi, IFS, PCCF (WL Division), Chief Wildlife Warden, Department of Forest Head Office, Govt. of Manipur, Sanjenthong, Imphal 795001 for permission No.3/22/2018-WL (Vol-II) to carry out herpetological surveys in Chandel District, Manipur. Financial support from funding agencies the Natonal Mission on Himalayan Studies (NMHS), Grant no: GBPNI/NMHS – 2017/MG – 22/566 and Department of Biotechnology, New Delhi, Government of India (Grant No: DBT-NER/AAB/64/2017). Note of special appreciation to David Huten, Monoel Sinruwng, Huten Peshumhring for companion in fieldwork. Developmental Biology and Herpetology Laboratory, Department of Zoology, Mizoram University, Tanhril, Aizawl, Mizoram, India.

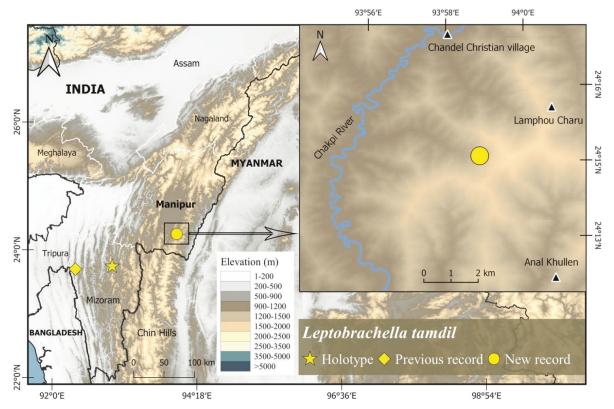


Figure 1. Map showing the type locality (in yellow star), previous record (in yellow diamond), and new record (in yellow dot) of *Leptobrachella* tamdil at Chakpi Stream, Chandel District, Manipur, India.

of 27-days field trip, and covering a total distance of ca. 120km. On 16 December 2020, we encountered the first two adult male individual frogs (MZMU 2224 and MZMU 2225) from the Chakpi Stream bed (24.1454N, 93.5856E; 1,122m) at around 17.40h, the sampling site is located ca. 30km south from Chandel Town, Chandel District, Manipur (Figure 1). The nearest village from the collection site is the Lamphou Charu located at ca. 4km. After a month, on 23 January 2021 at 19.59h, we encountered an adult female (MZMU 2226) at the upper stream bed (24.1453N, 93.5857E; 1,228m) ca. 7m away from the previous collection site. During the present work, the sampling sites were visited four times at day time, dusk, and night time.

Specimens of *L. tamdil* collected in this study were photographed by using Sony DSC-HX400V (50x optical zoom) digital camera. Specimens (MZMU 2224, MZMU 2225, and MZMU 2226) were fixed in 4% formalin, later preserved in 70% ethyl alcohol, liver tissues were stored in 95% ethyl alcohol for molecular processing, and deposited in Museum of Zoology, Mizoram University (MZMU), India. The altitude with the geo-locations was recorded by using global positioning system device (Garmin Montana 650-GPS navigator). A digital thermo hygrometer (Kusam Meco KM 918) was used for measurement of temperature and relative humidity. The morphometric parameters of the specimens were measured by using MitutoyoTM (505–730) dial calipers and are given to the nearest 0.1mm. The sex was determined through dissection. The parameters from Sengupta et al. (2010) and Vanlalsiammawii et al. (2020) were followed to measure the frog specimens (Table 1).

Genomic DNA was extracted from the 95% ethanol preserved liver tissues of the frog specimens using QIAamp DNA Mini Kit (Cat No.ID:51306) following the manufacturer protocol. PCR reaction was prepared for 20µL reaction mixture contained 1X amplification buffer, 2.5 mMMgCl₂, 0.25 mM dNTPs, 0.2 pM each forward and reverse primer, 1µL genomic DNA, and 1U Taq DNA polymerase with a pair of partial 16S rRNA primers: forward (L02510- CGC CTG TTT ATC AAA AAC AT) (Palumbi 1996) and reverse (H03063- CTC CGG TTT GAA CTC AGA TC) (Rassmann 1997). The PCR thermal regime for amplification was 5 min at 95°C for initial denaturation, followed by 35 cycles of 1 min at 95°C for denaturation, 30s for annealing at 50.3°C, elongation for 1 min at 72°C, and a final elongation for 5 min at 72°C. PCR products were checked by gel electrophoresis on a 1.5% agarose gel

Voucher				Vanlalsiammawii et al. (2020)	Sengupta e	et al. (2010)
number	MZMU2224	MZMU2225	MZMU2226	MZMU 1631	ZSI A10962 (Holotype)	ZSI A10963 (Paratype)
Sex	Male	Male	Female	Male	Male	Female
Locality		Chandel, Manipur		Dampa Tiger Reserve, Mizoram	Tamdil National V	Vetland, Mizoram
SVL	28.7	27.8	33.2	31.3	32.3	31.8
IN	2.7	2.7	2.9	3.2	3.2	3.1
HL	9.8	9.9	11.4	9.2	8.7	8.8
HW	9.6	9.2	11	10.5	12.0	12.0
HD	4.2	4	4.3	4.4	5.2	4.8
ED	4	4.2	4.8	4.3	4.5	4.6
10	3.9	3.8	4	4.8	5.1	5.8
E-S	3.5	3.9	4.4	4.6	4.7	4.7
E-N	2.1	1.7	2.2	2.5	2.8	2.7
UE	3	3.2	3.8	3.1	3.4	3.5
TL	12.6	12.7	15	14.2	16.0	15.7
IMT	1.8	1.5	1.8	1.8	1.9	1.8
IPT	1.8	1.5	2	2.1	2.2	1.8
A-G	14	13.6	16.3	13.7	13.8	13.8
BW	9.9	8.3	10.5	9.8	9.7	11.9
No. of eggs			n= 105			
Diameter of eggs			1.4–1.5			

Table 1. Detailed morphological (in mm) and eggs data of Leptobrachella tamdil from Chandel District, Manipur, India.

containing ethidium bromide. Samples were sequenced using Sanger's dideoxy method and sequencing reactions were carried out in both directions on a sequencer (Agrigenome Labs Pvt Ltd., Kochin, India). The generated partial 16S rRNA sequences were deposited in the GenBank repository (accession numbers: MW665130.1; MW665131.1; MW665132.1). In our dataset of 16S rRNA, we included 34 congeneric sequences obtained from National Centre for Biotechnology Information (NCBI) database and our generated sequence of Duttaphrynus melanostictus (MW165455.1) sample was used as an outgroup. All sequences were aligned by using Muscle algorithm in MEGA 7 (Kumar et al. 2016), the Kimura 2 (K2P) and genetic distances (Kimura, 1980) were calculated using MEGA 7 (Kumar et al., 2016). The Bayesian Inference (BI) phylogenetic tree (Figure 2) was constructed in MrBayes 3.2.5 using GTR+I+G model. The MCMC (one cold and three hot chains) was run for one million generations by sampling every 1,000 generations and set the burn-in to 25%. The analysis was terminated when the standard deviation of split frequencies was less than 0.001. The percentage of trees in which the associated taxa clustered together is shown next to the branches (Ronquist & Huelsenbeck 2003). The generated phylogenetic tree was further illustrated using Figtree 1.44v (Rambaut 2014).

RESULTS

The collected specimens are identified as L. tamdil based on the original morphological diagnostic features (Sengupta et al. 2010), and the new specimens showed genetic homogeneity on the phylogenetic tree with the mean intra-species K2P genetic distance of 0.0%. Our recently collected specimens are diagnosed in showing the following combination of characters: SVL between 27.8–28.7 mm in males and 33.2mm in the only female; dorsum tuberculate; eyelids with tubercles; tympanum and supratympanic fold distinct; supratympanic fold extending to posterior edge of tympanum; macroglands, including preaxillary, pectoral, femoral and ventrolateral glands present; Finger II > I; toe tips not dilated, bearing dermal fringes; relatively long hind limbs, with heels in contact when limbs are held perpendicular to body; dorsum with dark blotches; flanks with small dark blotches; dark tympanic mask present; venter pale; labial bars present and limbs with dark cross-bars (Sengupta et al. 2010). Current location extends the range of the species by ca. 122km aerial distance northeast from the

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										K2P	K2P distance										
	Species	1	2	m	4	2	9	7	∞	6	10	11	12	13	14	15	16	17	18	19	20
1	L. tamdil MW665131.1																				
2	L. tamdil MW665132.1	0.00													L						
m	L. tamdil MW665130.1	0.00	00.00																		
4	L. laui MH406903.1	0.09	60.0	0.09																	
ы	L. <i>liui</i> MH923370.1	0.09	0.09	0.09	0.04																
9	L. puhoatensis KY849587.1	0.09	0.09	0.09	0.07	60.0															
~	L. wulingensis MT530316.1	0.10	0.10	0.10	0.08	0.06	0.08														
∞	L. petrops MH055903.1	0.09	0.09	0.09	0.07	60.0	00.0	0.08													
6	L. bourreti KR018124.1	0.10	0.10	0.10	0.09	0.07	60.0	0.02	60.0												
10	L. minimus JN848369.1	0.11	0.11	0.11	0.07	0.08	60.0	0.08	60.0	0.09											
11	L. mangshanensis MH277365.1	0.10	0.10	0.10	0.05	0.02	0.09	0.08	0.09	0.08	0.08										
12	L. dorsospina MW046194.1	0.10	0.10	0.10	0.09	0.07	0.09	0.03	0.09	0.03	0.09	0.08									
13	L. nyx MH055818.1	0.10	0.10	0.10	0.08	0.07	0.09	0.07	0.09	0.08	0.06	0.07	0.08								
14	L. pluvialis MT644610.1	0.10	0.10	0.10	0.07	0.07	0.09	0.08	0.09	0.08	0.05	0.07	0.09	0.04							
15	L. purpuraventra MK414531.1	0.11	0.11	0.11	0.10	0.07	0.09	0.04	0.09	0.03	0.10	0.08	0.03	0.08	0.08						
16	L. suiyangensis MK829650.1	0.12	0.12	0.12	0.10	0.09	0.10	0.05	0.10	0.04	0.11	0.10	0.04	0.10	0.10	0.04					
17	L. alpinus MH406905.1	0.12	0.12	0.12	0.10	0.09	0.11	0.05	0.11	0.05	0.11	0.09	0.05	0.10	0.10	0.05	0.06				
18	L. shangsiensis MK095461.1	0.12	0.12	0.12	0.08	0.08	0.10	0.09	0.10	0.10	0.06	0.09	0.09	0.05	0.05	0.09	0.11	0.11			
19	L. aspera MW046202.1	0.12	0.12	0.12	0.10	0.10	0.10	0.08	0.10	0.09	0.08	0.10	0.10	0.06	0.06	0.10	0.11	0.11	0.06		
20	L. bijie MK414539.1	0.12	0.12	0.12	0.09	0.08	0.10	0.05	0.10	0.05	0.10	0.09	0.04	0.10	0.10	0.03	0.04	0.06	0.10	0.11	

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Occurrence of Leptobrachella tamdil in Manipur

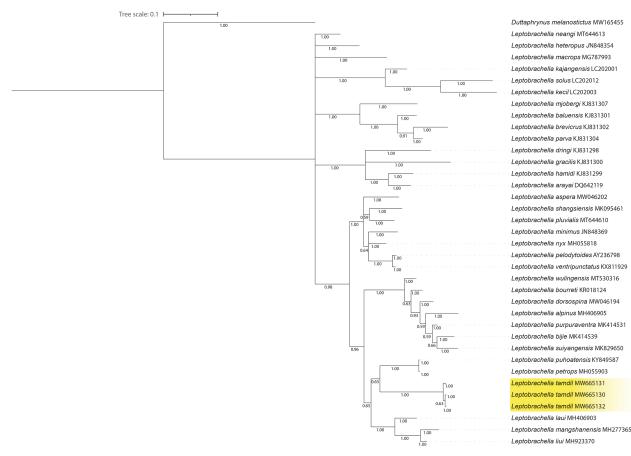


Figure 2. Bayesian inference phylogenetic relationship among Leptobrachella species using partial 16S rRNA gene sequences. The numbers at nodes represent Bayesian posterior probabilities.

type locality at Tamdil National Wetland, Mizoram, India.

Our generated partial 16S rRNA gene sequences of *L. tamdil* (MW665130.1; MW665131.1; MW665132.1) were compared with the congeners sequences obtained from NCBI database. From the estimated K2P genetic distances (Table 2), we infered that *L. laui* (MH406903.1) to be the closest species by showing 9% genetic distance with the sequences of *L. tamdil* (MW665130.1; MW665131.1; MW665132.1). Moreover, the phylogenetic relationship revealed that *L. tamdil* formed a distinct lineage within the monophyletic clade comprising *L. puhoatensis* + *L. petrops* (Figure 2); the former taxon (*L. puhoatensis*) is known only from its type locality in Pu Hoat Nature Reserve, Vietnam, and the later (*L. petrops*) is recorded only from four Provinces (Tuyen Quang, Lai Chau, Thanh Hoa, and Phu Tho) in Vietnam (Frost 2021).

DISCUSSION

Live individuals of *Leptobrachella tamdil* were found in the secondary forests, ca. 30km south of Chandel Town. Chandel District is surrounded by tropical semi-evergreen and moist deciduous/secondary forest, subjugated by Schima wallichii, Albizzia sp. and Macaranga denticulata. The forest in the moist valleys is lofty, while steep slopes are covered with canopy (Singh et al. 2000; Forest Survey of India 2019). Chakpi's slow-flowing streams where sampling was carried out, is surrounded by the subtropical semi-evergreen and the sub-tropical hill forests, predominantly subjugated by Juglans sp., Albizzia chinensis, Quercus sp., Macaranga denticulata, and Schima wallichi based on the classification of Champion & Seth (1968). Specimens were collected from beneath weathered sedimentary rocks and on the exposed sand stones in the vicinity close to slow-flowing stream. This is quite similar to the previous collection site of the species from Dampa Tiger Reserve, Mizoram State by Vanlalsiammawii et al. (2020). During the collection period, atmospheric temperature and relative humidity were 12.9°C and 83.6 %, respectively. Chakpi offshoots offer a unique ecosystem and congenial breeding grounds for many rare amphibian species especially near stagnant and flowing water. In Chakpi, streams were bounded by sedimentary rocks and weathered huge boulders and logs which provide suitable breeding spot for several



Image 1. Leptobrachella tamdil from Chandel District, Manipur State, India: A—Three adult individuals, left to right – (Male, MZMU2224; Male, MZMU 2225; Female, MZMU 2226) | B—Microhabitat at Chakpi Stream | C—Adult female individual MZMU 2226.

anuran species. Sympatric frog species includes Amolops cf. indoburmanensis and Sylvirana cf. lacrima that were observed at the upper reaches of the elevated stream bed. The present study found a gravid female (MZMU 2226) with 105 eggs. We suggest that the breeding season is likely to start during dry season (December to January) as hinted by the presence of gravid females and deposition of eggs. The egg diameter of L. tamdil range between 1.4-1.5 mm (N= 10). The conservation status for the species remains unclear. Deuti (2013) categorized this species as data deficient (DD), but later Dinesh et al. (2020) corrected that to not assessed (NA). Thus, the proper assessment of its conservation status is lacking. The microhabitat of *L. tamdil* consisted primarily of intermediate-flowing stream within tropical semievergreen forest (Sengupta et al. 2010; Vanlalsiammawii et al. 2020). Other aspects of L. tamdil such as the breeding biology, tadpole morphology, diet, and general

life history remain largely unknown and considerable works are needed to shed more light on this species. Legitimately, the present record of L. tamdil from northeastern part of India represents the northeasternmost locality with the highest altitude (1,220-1,228 m), against the records in Mizoram at 745 m (Sengupta et al. 2010) and 449 m (Vanlalsiammawii et al. 2020). This study provides a range extension of *L. tamdil* away from the type locality in Mizoram, north-east towards Manipur, and it is likely present in the adjacent country of Myanmar and possibly in Assam, Nagaland, and Tripura states. The new individuals represent the latest range of SVL (27.8–33.2 mm), and the breeding season might be commencing from late winter as indicated by the presence of gravid female. Further observations are necessary to know more information about the biology of L. tamdil.

The loss of forest canopy and natural streams were

Occurrence of Leptobrachella tamdil in Manipur

noted to directly threaten the habitats of the anuran species. Jhum cultivation, forestry effluents, and forest fires are also attributed to it (see Gupta 2000; Shimray 2004; Maithani 2005; Bhattacharya & Nanda 2005; Kerkhoff et al. 2006; Sastry et al. 2007; Jamir & Lianchawii 2013; Reimeingam 2017). The first step towards ensuring the long-term persistence of such anurans is addressing the lack of understanding of range, population trends, ecology, and potential threats. Mitigation measures must be put in place to stop the unchecked depletion of the resources of such little-known species, failing which L. tamdil and other such taxa will regrettably be wiped out from Manipur, to say the least (Banita & Bordoloi 2007). Overall, amphibian studies in the northeastern India is the least when compared to rest of the regions of the country and especially amphibian faunal inventorying is scanty in Manipur State. Safeguarding the ecological diversity of the existing areas is most likely to protect viable populations of such fragile wildlife. Surroundings of the Chandel Village include some intact habitats that are suitable for anurans where probable discovery of unique amphibians are reasonably high. Thus, extensive explorations can ascertain the true amphibian richness in the present study area.

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ISSN 0974-7907 (Online) | ISSN 0974-7893 (Print)

May 2021 | Vol. 13 | No. 6 | Pages: 18411–18678 Date of Publication: 26 May 2021 (Online & Print) DOI: 10.11609/jott.2021.13.6.18411-18678

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Conservation Application

First attempt at rehabilitation of Asiatic Black Bear cubs to the wild in Thailand – Robert Steinmetz, Worrapan Phumanee, Rungnapa Phoonjampa & Suthon Weingdow, Pp. 18411–18418

Communications

Status of Sumatran Tiger in the Berbak-Sembilang landscape (2020)

- Tomi Ariyanto, Yoan Dinata, Dwiyanto, Erwan Turyanto, Waluyo Sugito, Sophie Kirklin & Rajan Amin, Pp. 18419–18426

The diversity of small mammals in Pulau Perhentian Kecil, Terengganu, Malaysia – Aminuddin Baqi, Isham Azhar, Ean Wee Chen, Faisal Ali Anwarali Khan, Chong Ju Lian, Bryan Raveen Nelson & Javaraj Vijaya Kumaran, Pp. 18427–18440

Patterns, perceptions, and spatial distribution of human-elephant (*Elephas maximus*) incidents in Nepal

– Raj Kumar Koirala, Weihong Ji, Yajna Prasad Timilsina & David Raubenheimer, Pp. 18441–18452

Assessing spatio-temporal patterns of human-leopard interactions based on media reports in northwestern India

- Kaushal Chauhan, Arjun Srivathsa & Vidya Athreya, Pp. 18453-18478

Bat diversity in the Banpale forest, Pokhara, Nepal during spring season – Prabhat Kiran Bhattarai, Basant Sharma, Anisha Neupane, Sunita Kunwar & Pratyush Dhungana, Pp. 18479–18489

A patho-microbiological study of tissue samples of the Greater Adjutant *Leptoptilos dubius* (Aves: Ciconiiformes: Ciconiidae) that died in Deeporbeel Wildlife Sanctuary, Assam, India

– Derhasar Brahma, Parikshit Kakati, Sophia M. Gogoi, Sharmita Doley, Arpita Bharali, Biswajit Dutta, Taibur Rahman, Saidul Islam, Arfan Ali, Siraj A. Khan, Sailendra Kumar Das & Nagendra Nath Barman, Pp. 18490–18496

Vaduvur and Sitheri lakes, Tamil Nadu, India: conservation and management perspective – V. Gokula & P. Ananth Raj, Pp. 18497–18507

A new species of shieldtail snake (Squamata: Uropeltidae: Uropeltis) from the Bengaluru uplands, India

- S.R. Ganesh, K.G. Punith, Omkar D. Adhikari & N.S. Achyuthan, Pp. 18508-18517

A looming exotic reptile pet trade in India: patterns and knowledge gaps – A. Pragatheesh, V. Deepak, H.V. Girisha & Monesh Singh Tomar, Pp. 18518–18531

Legal or unenforceable? Violations of trade regulations and the case of the Philippine Sailfin Lizard Hydrosaurus pustulatus (Reptilia: Squamata: Agamidae) – Sarah Heinrich, Adam Toomes & Jordi Janssen, Pp. 18532–18543

Conservation breeding of Northern River Terrapin Batagur baska (Gray, 1830) in Sundarban Tiger Reserve, India

- Nilanjan Mallick, Shailendra Singh, Dibyadeep Chatterjee & Souritra Sharma, Pp. 18544–18550

Discovery of two new populations of the rare endemic freshwater crab Louisea yabassi Mvogo Ndongo, von Rintelen & Cumberlidge, 2019 (Brachyura: Potamonautidae) from the Ebo Forest near Yabassi in Cameroon, Central Africa, with recommendations for conservation action – Pierre A. Mvogo Ndongo, Thomas von Rintelen, Christoph D. Schubart, Paul F. Clark, Kristina von Rintelen, Alain Didier Missoup, Christian Albrecht, Muriel Rabone, Efole Ewoukem, Joseph L. Tamesse, Minette Tomedi-Tabi Eyango & Neil Cumberlidge, Pp. 18551–18558

Checklists of subfamilies Dryptinae and Panagaeinae (Insecta: Coleoptera: Carabidae) from the Indian subcontinent

- V.A. Jithmon & Thomas K. Sabu, Pp. 18559-18577

Mantids (Insecta: Mantodea) of Uttar Pradesh, India – Ramesh Singh Yadav & G.P. Painkra, Pp. 18578–18587

An assessment of genetic variation in vulnerable Borneo Ironwood *Eusideroxylon zwageri* Teijsm. & Binn. in Sarawak using SSR markers

– Siti Fatimah Md.-Isa, Christina Seok Yien Yong, Mohd Nazre Saleh & Rusea Go, Pp. 18588–18597

Review

Termites (Blattodea: Isoptera) of southern India: current knowledge on distribution and systematic checklist – M. Ranjith & C.M. Kalleshwaraswamy, Pp. 18598–18613

Short Communications

Population status and distribution of Ibisbill *Ibidorhyncha struthersii* (Vigors, 1832) (Aves: Charadriiformes: Ibidorhynchidae) in Kashmir Valley, India – Iqram Ul Haq, Bilal A. Bhat, Khursheed Ahmad & Asad R. Rahmani, Pp. 18614–18617

A new fish species of genus Garra (Teleostei: Cyprinidae) from Nagaland, India – Sophiya Ezung, Bungdon Shangningam & Pranay Punj Pankaj, Pp. 18618–18623

Occurrence of Tamdil Leaf-litter Frog Leptobrachella tamdil (Sengupta et al., 2010) (Amphibia: Megophryidae) from Manipur, India and its phylogenetic position – Ht. Decemson, Vanlalsiammawii, Lal Biakzuala, Mathipi Vabeiryureilai, Fanai Malsawmdawngliana & H.T. Lalremsanga, Po. 18624–18630

Further additions to the Odonata (Insecta) fauna of Asansol-Durgapur Industrial Area, Paschim Bardhaman, India

– Amar Kumar Nayak & Subhajit Roy, Pp. 18631–18641

A note on the ecology and distribution of Little Bloodtail Lyriothemis acigastra Brauer, 1868 (Insecta: Odonata: Libellulidae) in Kerala, India – Jeevan Jose, Muhamed Sherif & A. Vivek Chandran, Pp. 18642–18646

Viewpoint

A unique archetype of conservation in Himachal Pradesh, western Himalaya, India – Rupali Sharma, Monika Sharma, Manisha Mathela, Himanshu Bargali & Amit Kumar, Pp. 18647–18650

Notes

A camera trap record of Asiatic Golden Cat *Catopuma temminckii* (Vigors & Horsfield, 1827) (Mammalia: Carnivora: Felidae) in State Land Forest, Merapoh, Pahang, Malaysia – Muhamad Hamirul Shah Ab Razak, Kamarul Hambali, Aainaa Amir, Norashikin Fauzi, Nor Hizami Hassin, Muhamad Azahar Abas, Muhammad Firdaus Abdul Karim, Ai Yin Sow, Lukman Ismail, Nor Azmin Huda Mahamad Shubli, Nurul Izzati Adanan, Ainur Izzati Bakar, Nabihah Mohamad, Nur Izyan Fathiah Saimeh, Muhammad Syafiq Mohmad Nor, Muhammad Izzat Hakimi Mat Nafi & Syafiq Sulaiman. Pp. 18651–18654

Reappearance of Dhole *Cuon alpinus* (Mammalia: Carnivora: Canidae) in Gujarat after 70 years – A.A. Kazi, D.N. Rabari, M.I. Dahya & S. Lyngdoh, Pp. 18655–18659

Mating behavior of Eastern Spotted Skunk *Spilogale putorius* Linnaeus, 1758 (Mammalia: Carnivora: Mephitidae) revealed by camera trap in Texas, USA – Alexandra C. Avrin, Charles E.Pekins & Maximillian L. Allen, Pp. 18660–18662

Record of Indian Roofed Turtle Pangshura tecta (Reptilia: Testudines: Geoemydidae) from Koshi Tappu Wildlife Reserve, Nepal

- Ashmita Shrestha, Ramesh Prasad Sapkota & Kumar Paudel, Pp. 18663-18666

Additional distribution records of Zimiris doriae Simon, 1882 (Araneae: Gnaphosidae) from India – Dhruv A. Prajapati, Pp. 18667–18670

Notes on new distribution records of *Euaspa motokii* Koiwaya, 2002 (Lepidoptera: Lycaenidae: Theclinae) from Bhutan

- Jigme Wangchuk, Dhan Bahadur Subba & Karma Wangdi, Pp. 18671-18674

New distribution records of two little known plant species, *Hedychium longipedunculatum* A.R.K. Sastry & D.M. Verma (Zingiberaceae) and Mazus dentatus Wall. ex Benth. (Scrophulariaceae), from Meghalaya, India

- M. Murugesan, Pp. 18675-18678

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