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continued on the back inside cover

Caption: *Cyrtodactylus myintkyawthurai*, endemic to Myanmar. Medium: Water colours on watercolor sheet. © Aakanksha Komanduri

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Morphological characterization and mt DNA barcode of a tiger moth species, Asota ficus (Fabricius, 1775) (Lepidoptera: Noctuoidea: Erebidae: Aganainae) from India

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Abstract: The members of the genus Asota are widely distributed from Africa, India, Sri Lanka, Myanmar, and Malayan regions to the Australian region containing 55 described species. Asota ficus (Fabricius, 1775) is one among the nine species of the genus described from India having a wide range of distribution. The present study includes the first mitochondrial DNA barcode generated from India for A. ficus with a valid voucher describing external morphological characters together with the male and female genitalia. Discussions pertain to the utility of DNA barcodes for studies on moths in India with a comment on the identity of other sequences showing shallow genetic divergence with our sequences.

Keywords: Arctiinae, Ficus, genitalia study, Hypsa, Lepidopterism, Maharashtra, Mitragyna, molecular study, mt COI, Ricinus.

The subfamily Aganainae Boisduval, 1833 was earlier considered as family Aganaidae or Hypsidae (Inoue et al 1982). Later studies considered it as subfamily Hypsinae of Arctiidae (Seitz 1914; Daniel 1943) or subfamily Aganainae of Noctuidae (Holloway 1988; Scoble 1992; Kitching & Rawlins 1998). Until molecular studies, the familial position was unstable, later on phylogenetic studies placed the subfamily Aganainae under the family Erebidae (Fibiger & Lafontaine 2005; Zahiri et al. 2012).

Competing interests: The authors declare no competing interests.

Aganainae includes 109 species of 11 genera worldwide (Zahiri et al. 2012; Bayarsaikhan et al. 2016).

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Many Aganainae moths are large, brightly coloured, aposematic, with bare lower frons and long upturned labial palps having long and slender third segment; vein M2 in forewing arises closer to the origin of M3 than M1, in the lower part of the discal cell; Cu appearing four-branched; vein M2 in the hindwing is present so Cu appears four-branched (Holloway 1988; Zahiri et al. 2012). The larvae have single subventral seta on the mesothoracic and metathoracic segments. The subfamily exhibits a sister relationship with Arctiinae with a strongly supported pairing (Zahiri et al 2011).

Moths from this subfamily are pests on plant species of Apocynaceae, Asclepiadaceae, Moraceae (Holloway 1988; Common 1990; Bayarsaikhan et al. 2016), and lactiferous families that contain cardenolides (Bayarsaikhan et al. 2016). They feed on poisonous plants, and hence are often aposematic day flyers (Kitching & Rawlins 1998; Bayarsaikhan et al. 2016).

The genus Asota Hübner, [1819] was erected by Jacob

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Morphological characters and mt DNA barcode of Asota ficus

Hubner in 1819 considering Phalaena javana (Cramer, [1780]) from Java as type species. So far, 55 species are known from this genus including nine from India. The Asota species reported from India are: caricae (Fabricius, 1775); plana (Walker, 1854); canaraica (Moore, 1878); egens (Walker, 1854); ficus (Fabricius, 1775); heliconia (Linnaeus, 1758); paphos (Fabricius, 1787); producta (Butler, 1875); sericea (Moore, 1878). A. ficus was placed under the genus Hypsa as Hypsa ficus by Hampson (1892) under the family Hypsidae: section-II. Hampson (1892) divided the genus Hypsa under two sections on the basis of structure of antennae. In Section-I the antennae of males are fasciculated with short cilia. The fasciculated male antennae, long cilia and the long 3rd segment of palpi forms the section-II. Caterpillar of A. ficus is recorded feeding mainly on castor and ficus.

The genus *Asota* is responsible for Lepidopterism, a disease caused by the adult or the caterpillar of moths or butterflies (Wills et al. 2016). In Kerala India, it was reportedly caused by the tiger moth *A. caricae* (Anonymous 2016). The fever caused by Lepidopterism mimics the symptoms of the mosquito borne infectious diseases like chikungunya and dengue. The adult moths, while emerging from the pupae, extricate the scales on their body and secretes fluids (Anonymous 2016) which lead to the high fever either when in contact with the human skin or due to inhalation. As per Wills et al. (2016), allergic reactions are due to the presence of poisonous chemicals like histamines, imidazole and peptides.

DNA barcoding is a quick and reliable nucleotidebased identification technique across the animal kingdom, founded on the mitochondrial Cytochrome oxidase I gene (mt COI) by Hebert's group in 2003. The ability of COI sequences to discriminate closely allied species based on restricted intraspecific mitochondrial DNA divergence and utilizing it as an aid to resolve the alpha diversity of species in diverse taxonomic groups including Lepidoptera has been validated (Hebert et al. 2003b). These species-specific signatures, identified as DNA barcodes help to delimit the problematic taxa (Hebert et al. 2003a) also in cases where identification is not possible with the traditional taxonomic techniques alone. DNA barcode not only provides a boon to taxonomic research but also serves as a form of comprehensive, widely accessible system for identification and validation of species. Hence, in the present study an attempt has been made to develop a DNA barcode for the species A. ficus from Maharashtra along with its morphological description (adult together with external genitalia); the utility of mt DNA barcodes in the Indian moth studies are discussed.

MATERIALS AND METHODS

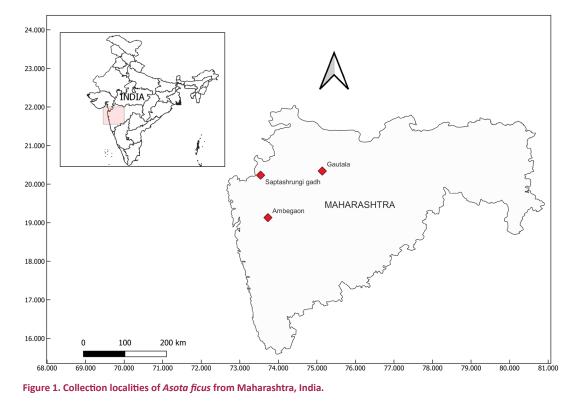
Moth specimens were collected using a light trap having mercury vapour lamp as a light source of 160 W. It was hung in the middle of the white sheet installed in the field during the night. Moth specimens that were captured were euthanized by ethyl acetate vapours. Then they were transported to the laboratory in insect packets (made of butter paper) for further analysis.

In the laboratory, the specimens were stretched, pinned and stored in entomological boxes filled with preservatives. For morphological studies the specimens were studied under Leica EZ4E stereomicroscope. The map of the collection locality was prepared using open free QGIS software. The details of the collection locality are given under the material examined and is also shown in Figure 1. Identification of the specimens was done as per Hampson (1892). Male and female genitalia were studied following Robinson (1976). The identified specimens are deposited at the National Zoological Collections of the Zoological Survey of India, Western Regional Centre, Pune, Maharashtra, India (ZSI/WRC).

DNA extraction was performed using DNeasy blood and tissue kit (Qiagen) using leg and abdomen of a dried specimen. DNA quantitation was performed by HS dsDNA assay kit on Qubit 2.0 fluorometer. Mitochondrial COI (mt COI) gene was amplified using universal primer pair, LCO1490 and HCO2198 (Folmer et al. 1994) in 25 µL reaction volume constituted by 12.5 µL of Master Mix (Promega), 10 pmol of each forward and reverse primer, 50 ng of template DNA along with Nuclease free water up to Q.S. Thermal cycling profile performed as per Kalawate et al. (2020a). Amplification of the desired gene was confirmed by gel electrophoresis stained by SYBR safe DNA gel stain (Invitrogen), visualized under UV by gel documentation system. Purification of the amplified product was done by Invitrogen's Pure Link PCR Purification Kit. The purified PCR product was sequenced bi-directionally by Sanger's method on ABI 377 (Applied Biosciences) sequencer.

Both the forward and reverse sequences generated in the current studies were verified manually for corrections. Initially 838 mt COI gene sequences available for the genus *Asota* were downloaded from the GenBank and were aligned using MEGA 5.2 software (Tamura et al 2011). MEGA 5.2 (Tamura et al. 2013) was used for calculating uncorrected pairwise genetic distances. Initial tree was built (using MEGA 5.2) including all reported species with molecular data for the genus *Asota*, comprising 235 sequences excluding identical sequences from the same locality for a single species/subspecies. Since mt COI is not a good candidate

Kalawate et al.



gene for phylogenetic studies (Cameron et al. 2004; Lafontaine & Schmidt 2010) and our initial single gene phylogenetic tree ended up in polytomies without proper phylogenetic relationships, we considered presenting the phylogenetic tree comprising all the sequences of A. ficus available on the GenBank with the sequences generated by us and the probable sister species A. speciosa treating species Neochera inops as an outgroup. The phylogenetic inferences drawn are only to show the monophyly of all the sequences of A. ficus. Maximum likelihood tree was generated using RaxML (Silvestro & Michalak 2012) with thorough bootstrap of 1,000 replicates under the GTR+GAMMA+I model and the final consensus tree was visualized by Fig Tree v1.4.0. Sequences generated in the studies are submitted to the GenBank (OL630456.1 & OL630457.1).

RESULT AND DISCUSSIONS

Taxonomic account

Superfamily Noctuoidea Latreille, 1809 Family Erebidae Leach, [1815] Subfamily Aganainae Boisduval, 1833 Genus *Asota* Hübner, [1819] *Asota* Hübner, [1819], *Verz. bek. Schmett*. (11): 164. Type Species: *Phalaena javana* (Cramer, [1780])

Asota ficus (Fabricius, 1775)

Noctua ficus Fabricius,1775, Syst. Ent.: 595. Lacides ficus, Moore,188, Lep. Ceylon, 2(1): 53, pl. 100,

f. 2.

Hypsa ficus, Hampson, 1892, *Fauna Brit. India, Moths*, 1: 504.

Type Locality. India.

Material examined/source: 01 male, Saptashringigadh, Nashik, Maharashtra, India (20.23N, 73.54E; 1,000 m), 06 November 2016, coll. A.S. Kalawate (ZSI/WRC/L-1482); 01 female, Ambegaon, Pune, Maharashtra, India (19.13N, 73.73E; 730 m), 23 June 2017, coll. A.S. Kalawate & party (ZSI/WRC/L-1780); 02 male, Bhaskaracharya Forest Rest house, Gautala, Jalgaon, Maharashtra, India (20.34N, 75.14E; 711 m), 27 September 2019, coll. P.S. Bhatnagar & party (ZSI/ WRC/L-2069).

Morphological description: Adult (Image 1A,B). Wing expanse: 55 mm in male and 63 mm in female. Antennae of male fasciculated, cilia long; 3rd joint of palpi long, grey in colour, tipped with black. Head, thorax and abdomen orange-yellow; tegulae with yellow base and a black spot. Abdomen with series of black spots. Orange basal patch on forewing extending along costa and in cell to two-third length of cell, an orange spot encircled with black on the costa, and streaks in cell and on inner margin, two black spots on costa and in

Kalawate et al.

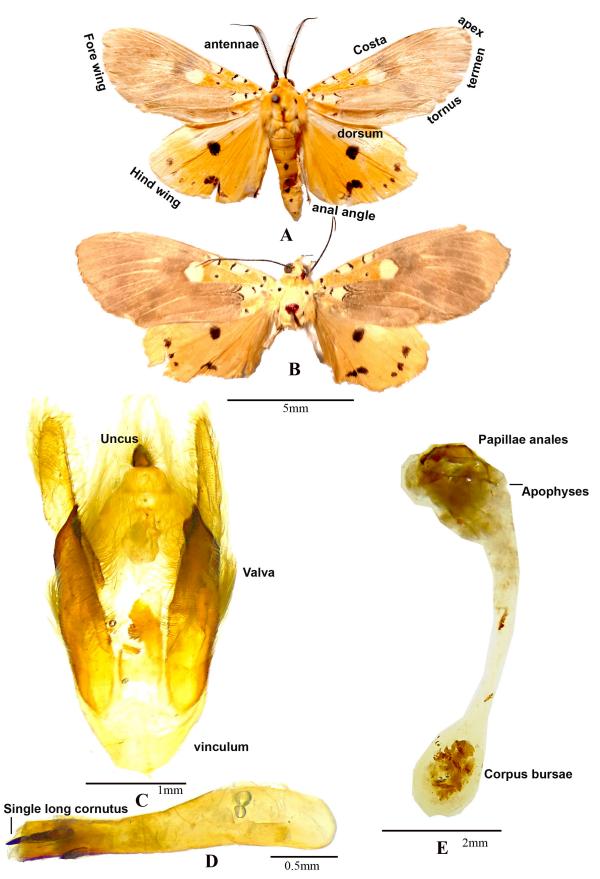
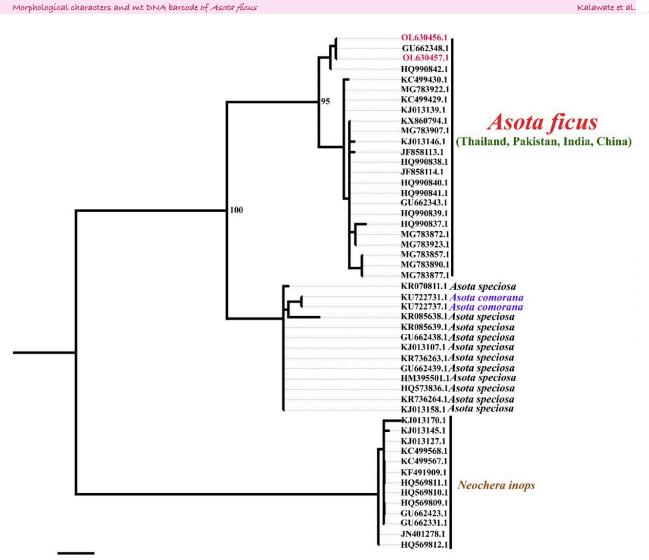


Image 1. Asota ficus: A-Male | B-Female | C-Genitalia | D-Aedeagus | E-Female genitalia.



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Figure 2. Maximum likelihood (ML) tree for the species of Asota based on the 578 bp of mitochondrial COI DNA gene sequences.

cell, one on inner margin, and two lines across internomedian interspace; rest of the wing olive-brown, the veins are striped with yellow. Hind wing bright orangeyellow; black spot at end of cell and series of irregular sized and placed black spots at submarginal area. Male and female are similar in external morphology except antennae. In male they are, fasciculated with long cilia and very short cilia in female.

Male genitalia (Image 1C). Uncus long, highly sclerotised broad till middle and then narrowing down, apex pointed recurved. Tegumen longer than the uncus, moderately sclerotised with broad arms, inverted v-shaped; valvae symmetrical, weakly sclerotised, setosed, costa strongly produced into a long process, harpe with a pointed process; vinculum longer than tegumen, u-shaped; juxta elongated; Aedeagus (Image 1D) long, relatively thin, apical portion dentate ventrally. Vesica membranous with single, long cornutus.

Female genitala (Image 1E). Corpus bursae oblong, membranous; ductus bursae long, membranous; ostium bursae simple, sclerotized; posterior and anterior apophyses are of equal length, sclerotized; papilla analis oval, heavily sclerotized with setae.

Distribution: India (throughout including Maharashtra), China, Japan, Malaysia, Myanmar, Nepal, Sri Lanka, Taiwan, and Thailand.

Host plants. *Ricinus communis, Ficus carica, F. hispida, F. racemosa, F. pumila, F. infectoria, F. religiosa,* and *Mitragyna diversifolia* (ICAR-NBAIR 2020).

DNA barcode studies: In the GenBank a total of 22 sequences of mt COI are available for *A. ficus* (Table 1), of which nine sequences are from India. Within India, these sequences are from the states of Assam, Maharashtra and Tamil Nadu (all are unpublished data

Table 1. Details of the mt COI GenBank accession numbers of Asota utilised in the construction of ML phylogenetic tree.

	GenBank Accession No.	Locality	Species name as per NCBI	Publication details as per NCB
1	GU662348.1	Thailand: Chiang Mai	Asota ficus	Unpublished
2	OL630456.1	India: Maharashtra, Nasik, Saptashrungigadh.	Asota ficus	Current study
3	OL630457.1	India: Maharashtra , Jalgaon	Asota ficus	Current study
4	HQ990842.1	Pakistan	Asota ficus	Unpublished
5	KC499430.1	India: Tamil Nadu, Kalkad	Asota ficus	Unpublished
6	MG783922.1	India: Maharashtra	Asota ficus	Unpublished
7	KC499429.1	China: Yunnan	Asota ficus	Unpublished
8	KJ013139.1	India: Assam,	Asota ficus	Unpublished
9	KX860794.1	Pakistan: Punjab	Asota ficus	Ashfaq et al. (2017)
10	MG783907.1	India: Maharashtra	Asota ficus	Unpublished
11	KJ013146.1	India: Nameri NP	Asota ficus	Unpublished
12	JF858113.1	Pakistan	Asota ficus	Unpublished
13	HQ990838.1	Pakistan	Asota ficus	Unpublished
14	JF858114.1	Pakistan	Asota ficus	Unpublished
15	HQ990840.1	Pakistan	Asota ficus	Unpublished
16	HQ990841.1	Pakistan	Asota ficus	Unpublished
17	GU662343.1	Thailand: Chiang Mai	Asota ficus	Unpublished
17	HQ990839.1	Pakistan	Asotaficus	Unpublished
19	HQ990837.1	Pakistan	Asota ficus	Unpublished
20	MG783872.1	India: Maharashtra	Asota ficus	Unpublished
20	MG783923.1	India: Maharashtra	Asota ficus	Unpublished
21	MG783857.1	India: Maharashtra	Asota ficus	Unpublished
22	MG783890.1	India: Maharashtra	-	
			Asota ficus	Unpublished
24	MG783877.1	India: Maharashtra	Asota ficus	Unpublished
25	KR070811.1	Kenya: Kajiado North	Asota speciosa	Unpublished
26	KU722731.1	Comoros: Grande Comore	Asota comorana	Unpublished
27	KU722737.1	Comoros: Grande Comore	Asota comorana	Unpublished
28	KR085638.1	Zambia: Victoria Falls	Asota speciosa	Unpublished
29	KR085639.1	Zambia: Lusaka Ridgeway	Asota speciosa	Unpublished
30	GU662438.1	Nigeria: Laeinde	Asota speciosa	Unpublished
31	KJ013107.1	Tanzania: Mbizi forest	Asota speciosa	Unpublished
32	KR736263.1	Nigeria:Oyo	Asota speciosa	Unpublished
33	GU662439.1	Cameroon: North Province	Asota speciosa	Unpublished
34	HM395501.1	Gabon: WoleuNamiTchimble	Asota speciosa	Unpublished
35	HQ573836.1	Gabon: Ogooue-Ivindo	Asota speciosa	Unpublished
36	KR736264.1	Nigeria:Oyo	Asota speciosa	Unpublished
37	KJ013158.1	Ethiopia: Arba Minch	Asota speciosa	Unpublished
38	KJ013170.1	Laos: Nang Phoa	Neochera inops	Unpublished
39	KJ013145.1	Laos: Nang Phoa	Neochera inops	Unpublished
40	KJ013127.1	Laos: Namha protected area,	Neochera inops	Unpublished
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43	KF491909.1	Malaysia	Neochera inops	Unpublished
44	HQ569811.1	Thailand: Nan	Neochera inops	Unpublished
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46	HQ569809.1	VietNam: Tam Dao	Neochera inops	Unpublished
47	GU662423.1	Thailand: Chiang Mai	Neochera inops	Unpublished
48	GU662331.1	Thailand: Chiang Mai	Neochera inops	Unpublished
49	JN401278.1	Japan	Neochera inops	Zahiri et al. (2012)
50	HQ569812.1	Malaysia: Sarawak	, Neochera inops	Unpublished

as per GenBank). The current study forms the first published record of DNA barcode for the species *A. ficus* from India with assigned voucher numbers.

In the preliminary phylogenetic tree generated for the studies, all the mt DNA barcodes formed a monophyletic clade for the species A. ficus (Figure 2) showing genetic distance variance from 0.6% to 1.3%. The clade comprising A. speciosa and A. comorana showed sister relationship with the clade of A. ficus, wherein genetic distance between the species A. ficus and A. comorana was 2.9% and A. ficus and A. speciose was 3.4%. In the present study A. comorana is nested within A. speciosa which suggests either one of the species was wrongly identified ending up in mislabelled sequences or synonymy of these two taxa. Further studies are necessary to resolve the identity and validity of the species A. comorana as the genetic distance between the species A. speciosa and A. comorana is too shallow (0.6–1.7 %).

Evolutionary distances are fundamental in molecular reconstructions including phylogenetic analysis (Nei & Kumar 2000). The nucleotide substitution method is widely used to calculate a reliable genetic difference between pairs of sequences (Nei & Kumar 2000). Since there are limitations with the mt COI gene (Cameron et al. 2004; Hebert & Gregory 2005; Lafontaine & Schmidt 2010), we suggest further studies to comment on the phylogenetic relationships among the species of the genus *Asota*. Nuclear DNA (n DNA) studies are advocated (Zahiri et al. 2012) to study ancient evolutionary divergence for resolving deeper nodes above species level, having slower mutation rate than mt DNA.

In India, generation of mt COI DNA barcodes for moths is still in a stage of infancy. Recently, Kalawate et al (2020a) have reported the palearctic moth species Olepa schleini Witt et al. 2005 from India with a description of subspecies based on the DNA barcode studies and morphological variations. Additionally, Kalawate et al. (2020b) described three new species along with a subspecies and provided the description of multiple morphotypes of Olepa from India. These studies clearly endorse the utility of DNA barcodes in identification of palearctic species from India (Kalawate et al. 2020a). This technique further avoids taxonomic inflation by describing morphologically different looking morphotypes as a new species (Kalawate et al. 2020b). Further, DNA barcode studies are expected to alleviate identification of morphologically variant species and uncover the cryptic diversity prevailing within the taxonomic groups. Multigene phylogenetic analysis is warranted to decipher the phylogenetic relationships across the members of the family which are wide spread in distribution range.

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20509

Morphological characters and mt DNA barcode of Asota ficus

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Articles

Estimating the completeness of orchid checklists and atlases: a case study from southern Italy

- Antonio Croce, Pp. 20311-20322

A floristic survey across three coniferous forests of Kashmir Himalaya, India – a checklist

Ashaq Ahmad Dar, Akhtar Hussain Malik & Narayanaswamy Parthasarathy, Pp. 20323–20345

Associations of butterflies across different forest types in Uttarakhand, western Himalaya, India: implications for conservation planning – Arun Pratap Singh, Pp. 20346–20370

Comparison of bird diversity in protected and non-protected wetlands of western lowland of Nepal

– Jagan Nath Adhikari, Janak Raj Khatiwada, Dipendra Adhikari, Suman Sapkota, Bishnu Prasad Bhattarai, Deepak Rijal & Lila Nath Sharma, Pp. 20371–20386

Local hunting practices and perceptions regarding the distribution and ecological role of the Large Flying Fox (Chiroptera: Pteropodidae: *Pteropus vampyrus*) in western Sarawak, Malaysian Borneo

– Jayasilan Mohd-Azlan, Joon Yee Yong, Nabila Norshuhadah Mohd Hazzrol, Philovenny Pengiran, Arianti Atong & Sheema Abdul Aziz, Pp. 20387–20399

Communications

20469-20477

Macrolichens of Mathikettan Shola National Park, Western Ghats: a preliminary investigation with some new records

- Aswathi Anilkumar, Stephen Sequeira, Arun Christy & S.M. Arsha, Pp. 20400-20405

New distribution record of globally threatened Ocean Turf Grass Halophila beccarii Ascherson, 1871 from the North Andaman Islands highlights the importance of seagrass exploratory surveys

– Swapnali Gole, Prasad Gaidhani, Srabani Bose, Anant Pande, Jeyaraj Antony Johnson & Kuppusamy Sivakumar, Pp. 20406–20412

An inventory of new orchid (Orchidaceae) records from Kozhikode, Kerala, India – M. Sulaiman, C. Murugan & M.U. Sharief, Pp. 20413–20425

Abundance and spatial distribution analyses of *Stemonoporus moonii* Thwaites (Dipterocarpaceae) - a critically endangered species endemic to Sri Lanka – K.A.M.R.P. Atapattu, H.D.D.C.K. Perera, H.S. Kathriarachchi & A.R. Gunawardena, Pp. 20426–20432

Plant diversity of Point Calimere Wildlife Sanctuary and fodder species grazed by the Blackbuck Antilope cervicapra L.

Ashutosh Kumar Upadhyay, A. Andrew Emmanuel, Ansa Sarah Varghese & D. Narasimhan, Pp. 20433–20443

Raptors observed (1983–2016) in National Chambal Gharial Sanctuary: semi-arid biogeographic region suggestions for parametric studies on ecological continuity in Khathiar-Gir Ecoregion, India – L.A.K. Singh, R.K. Sharma & Udayan Rao Pawar, Pp. 20444–20460

Nesting success of Sharpe's Longclaw (*Macronyx sharpei* Jackson, 1904) around the grasslands of lake Ol'bolossat Nyandarua, Kenya – Hamisi Ann Risper, Charles M. Warui & Peter Njoroge, Pp. 20461–20468

Population, distribution and diet composition of Smooth-coated Otter Lutrogale perspicillata Geoffroy, 1826 in Hosur and Dharmapuri Forest Divisions, India – Nagarajan Baskaran, Raman Sivaraj Sundarraj & Raveendranathanpillai Sanil, Pp.

Utilization of home garden crops by primates and current status of human-primate interface at Galigamuwa Divisional Secretariat Division in Kegalle District, Sri Lanka

 Charmalie Anuradhie Dona Nahallage, Dahanakge Ayesha Madushani Dasanayake, Dilan Thisaru Hewamanna & Dissanayakalage Tharaka Harshani Ananda, Pp. 20478– 20487 Revival of Eastern Swamp Deer Rucervus duvaucelii ranjitsinhi (Groves, 1982) in Manas National Park of Assam, India

– Nazrul Islam, Aftab Ahmed, Rathin Barman, Sanatan Deka, Bhaskar Choudhury, Prasanta Kumar Saikia & Jyotishman Deka, Pp. 20488–20493

Trypanosoma evansi infection in a captive Indian Wolf *Canis lupus pallipes* – molecular diagnosis and therapy

– Manojita Dash, Sarat Kumar Sahu, Santosh Kumar Gupta, Niranjana Sahoo & Debarat Mohapatra, Pp. 20494–20499

View Point

COVID-19 and civil unrest undoing steady gains in karst conservation and herpetological research in Myanmar, and an impediment to progress

– Evan S.H. Quah, Lee L. Grismer, Perry L. Wood, Jr., Aung Lin & Myint Kyaw Thura, Pp. 20500–20502

Short Communications

Morphological characterization and mt DNA barcode of a tiger moth species, *Asota ficus* (Fabricius, 1775) (Lepidoptera: Noctuoidea: Erebidae: Aganainae) from India – Aparna Sureshchandra Kalawate, K.P. Dinesh & A. Shabnam, Pp. 20503–20510

Distribution of Smooth-coated Otters *Lutrogale perspicillata* (Mammalia: Carnivora: Mustelidae): in Ratnagiri, Maharashtra, India – Swanand Patil & Kranti Yardi, Pp. 20511–20516

Wildlife at the crossroads: wild animal road kills due to vehicular collision on a mountainous highway in northwestern Himalayan region

– Muzaffar A. Kichloo, Asha Sohil & Neeraj Sharma, Pp. 20517–20522

Notes

Robiquetia gracilis (Lindl.) Garay—a new record to the flora of Anamalai Hills, Tamil Nadu, India

– B. Subbaiyan, V. Ganesan, P.R. Nimal Kumar & S. Thangaraj Panneerselvam, Pp. 20523–20525

Ipomoea laxiflora H.J. Chowdhery & Debta (Convolvulaceae): new records for the Western Ghats and semiarid regions

- Sachin M. Patil, Ajit M. Vasava, Vinay M. Raole & Kishore S. Rajput, Pp. 20526-20529

Counting the cost: high demand puts Bunium persicum (Boiss.) B.Fedtsch. in jeopardy

- Monika Sharma, Manisha Mathela, Rupali Sharma, Himanshu Bargali, Gurinderjit S. Goraya & Amit Kumar, Pp. 20530–20533

First record of Parasitic Jaeger *Stercorarius parasiticus* (Aves: Charadriiformes: Stercorariidae) from inland freshwater Inle Lake, Myanmar

– Sai Sein Lin Oo, Myint Kyaw, L.C.K. Yun, Min Zaw Tun, Yar Zar Lay Naung, Soe Naing Aye & Swen C. Renner, Pp. 20534–20536

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