



Confirmation of Irawadi Forest Lizard, *Calotes irawadi* Zug, Brown, Schulte and Vindum 2006 (Squamata: Agamidae), from Manipur, India

Ht Decemson¹, Ronald Rohluosang Sinate², Lal Biakzuala¹, Mathipi Vabeiryureilai¹, Paul Lalnunluanga³, Jehova Malsawma³, and Hmar Tlawmte Lalremsanga¹

¹Developmental Biology & Herpetology Laboratory, Department of Zoology, Mizoram University, Aizawl – 796004, Mizoram, India (htlr@aahoo.co.in)

²Muolbem, Churachandpur – 795128, Manipur, India

³Department of Geology, Mizoram University, Aizawl – 796004, Mizoram, India

At present, the agamid genus *Calotes* Cuvier 1817 consists of 29 species, of which 12 species are recorded in India and three species are known to occur in the state of Manipur; specifically: *C. emma* Gray 1835, *C. geissleri* Wagner, Ihlow, Hartmann, Flecks, Schmitz and Böhme 2021, and *C. versicolor* (Daudin 1802) (Matthew 2005; Decemson et al. 2021; Uetz et al. 2022). We report the occurrence of the Irawadi Forest Lizard, *C. irawadi* Zug, Brown, Schulte, and Vindum 2006 in Manipur. Historically the species was known from its type locality in Sagaing Division, Myanmar (Zug et al. 2006), Western Yunnan in China (Liu et al. 2021), and Arunachal Pradesh, Tripura, and Mizoram in northeastern India (Gowande et al. 2021; Tariang et al. 2022). Although Matthew (2005) reported *C. versicolor* from various parts of Manipur, there has not been a taxonomic re-assessment of these individuals in the state since the revision of the species

complex by Gowande et al. (2021). To verify the taxonomic identity of the *C. “versicolor”* population in Manipur, we used morphology and molecular analysis to confirm the identity of *Calotes* in Manipur.

On 12 December 2021, a road-killed adult male *Calotes* sp. was collected near Sinzawl village, Churachandpur District, Manipur, India (24°04'01" N; 93°18'14" E; 821 m asl; Fig. 1A&B). It was later catalogued in the Departmental Museum of Zoology, Mizoram University (MZMU), and diagnostic characters from Zug et al. (2006) were used to confirm the species identity. We determined sex by dissecting the tail-base to detect the presence or absence of hemipenes. We documented another individual from the same locality, but the lizard escaped after taking a photograph (Fig. 1C).

Prior to formalin fixation of the specimen (MZMU2230), we collected liver tissue for molecular analysis. We extracted

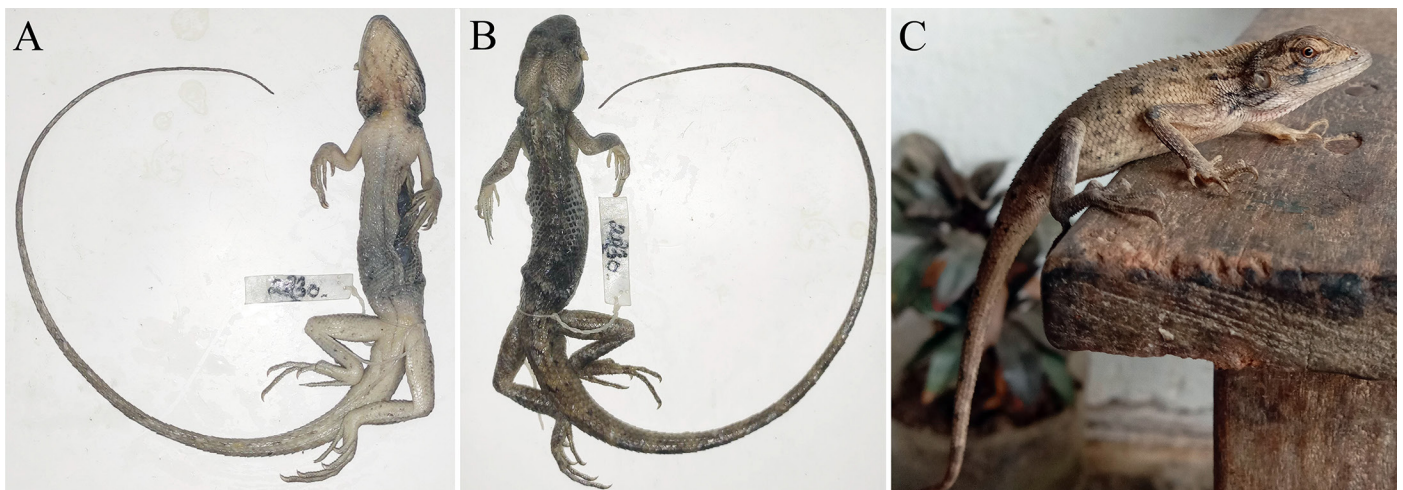


Fig. 1. *Calotes irawadi* from Manipur, India: (A) ventral view and (B) dorsal views of the preserved specimen (MZMU2230), (C) uncollected individual. Photographs for 1A&B by Ht. Decemson; photograph for 1C by Ronald Rohluosang Sinate.

genomic DNA (gDNA) from the liver tissue using QIAamp DNA Mini Kit (Qiagen Cat. No. 51306) following the manufacturer’s protocol. We did a polymerase chain reaction (PCR) using a 20µL reaction mixture containing 1X amplification buffer, 2.5 mM MgCl₂, 0.25mM dNTPs, 0.2pM 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). To amplify PCR product, we used the following thermocycler protocol: 5 min at 95 °C for initial denaturation, followed by 35 cycles of 1 min at 95 °C for denaturation, 30 s for annealing at 50.3 °C, elongation for 1 min at 72 °C, and a final elongation for 5 min at 72 °C. We checked PCR product using gel electrophoresis on a 1.5% agarose gel containing ethidium bromide. We sequenced samples using Sanger methods in both forward and reverse directions (Barcode Bioscience, Bangalore, India).

For the molecular analysis, we compiled a dataset consisting of our newly generated sequence, fourteen congener sequences obtained from NCBI GenBank and one sequence of *Monileosaurus rouxii* (MH844748) that was used as an outgroup (Table 1). The assembled dataset was aligned using the MUSCLE algorithm (Edgar 2004) with the default

parameter settings in MEGA 7 (Kumar et al. 2016). The final aligned dataset contains 490 basepairs. The Kimura 2 parameter (K2P) genetic distance (Kimura 1980) was estimated in MEGA 7 (Kumar et al. 2016). The best-fitting nucleotide evolutionary model (GTR+G) was selected in Modeltest-NG (Darriba et al. 2020) using the Bayesian Information Criterion (BIC). We estimated the Bayesian Inference (BI) phylogenetic tree in MrBayes 3.2.5 (Ronquist and Huelsenbeck 2003) using GTR+G substitution model. The Markov Chain Monte Carlo (MCMC) was run for 10,000,000 generations using one cold and three hot chains and sampling the posterior every 500 generations. To determine nodal, we consider nodes with a posterior probability (PP) ≥ 0.95 to be well supported. We discarded the first 25% of posterior samples as burn-in. When the standard deviation of split frequencies became less than 0.01, we terminated the analysis. We reconstructed the Maximum Likelihood (ML) phylogeny using in RAxML 2.0 (Edler et al. 2021). To assess nodal support, we used 1,000 rapid bootstraps (RBS). We visualized both phylogenetic trees using Figtree v1.4.4.

We identified the new specimen (MZMU2230) from Manipur as *C. irawadi* based on diagnostic morphological characters from Zug et al. (2006) and descriptions given by Liu et al. (2021). Here we provide a description of morpho-

Table 1. Specimens used for phylogenetic estimation in this study

Sl. No.	Species	Specimen vouchers	NCBI Accession Number	Grid references	Locality	References
1	<i>Calotes irawadi</i>	MZMU2230	ON041215	24.066944°N, 93.303889°E	Manipur, India	This study
2	<i>Calotes irawadi</i>	MZMU2385	OL691118	23.73772°N, 92.66773°E	Mizoram, India	Tariang et al. 2022
3	<i>Calotes irawadi</i>	MZMU2596	OL691119	23.83348°N, 92.74365°E	Mizoram, India	Tariang et al. 2022
4	<i>Calotes irawadi</i>	AG 855	MW901300	23.5743°N, 95.7376°E	Tripura, India	Gowande et al. 2021
5	<i>Calotes irawadi</i>	CAS 204862	MW901301	23.5743°N, 95.7376°E	Sagaing, Myanmar	Gowande et al. 2021
6	<i>Calotes irawadi</i>	CESL 1089	MW901302	23.5743°N, 95.7376°E	Arunachal Pradesh, India	Gowande et al. 2021
7	<i>Calotes versicolor</i>	L182C	MH844729	9.97136°N, 77.2328°E	Kerala, India	Gowande et al. 2016
8	<i>Calotes versicolor</i>	L190C	MH844730	11.9664°N, 79.7633°E,	Tamil Nadu, India	Gowande et al. 2016
9	<i>Calotes emma</i>	NCBSAQ	MH844707	—	India	Pal et al. 2018
10	<i>Calotes emma</i>	NCBS	MK789847	—	Meghalaya, India	Giri et al. 2019
11	<i>Calotes farooqi</i>	DJ 10247	MW901314	—	Pakhtunkhwa, Pakistan	Gowande et al. 2021
12	<i>Calotes grandisquamis</i>	L035C	MH844712	—	Kerala, India	Pal et al. 2018
13	<i>Calotes mystaceus</i>	WII	MK789848	—	Nagaland, India	Giri et al. 2019
14	<i>Calotes nemoricola</i>	L555C	MH844745	—	Pushpagiri Wildlife Sanctuary, Karnataka, India	Pal et al. 2018
15	<i>Calotes calotes</i>	L374C	MH844738	—	Kerala, India	Pal et al. 2018
16	<i>Monileosaurus rouxii</i>	L834C	MH844748	—	Kudremukh National Park, Karnataka, India	Pal et al. 2018

metric characters for MZMU2230 and compare them to the published range of each character in parentheses. Snout-vent length 86.4 mm (64.3–106.8 mm); head scales 12 (10–15); 4th finger lamellae 21 (17–24); 4th toe lamellae 24 (22–29); dorsal scales 54 (36–59) and midbody scales 45 (40–51).

Both the BI and ML phylogenetic analyses revealed similar tree topologies, and largely reflect interspecific relationships within *Calotes* recovered by Zug et al. (2006) and Giri et al. (2019). Our sample of *C. irawadi* from Manipur is clearly nested among individuals of *C. irawadi* recently confirmed by Gowande et al. (2021) and Tariang et al. (2022). Individuals of *C. irawadi* from India and Myanmar cluster to form a well-supported monophyletic clade to the exclusion of *C. calotes*, *C. versicolor* and *C. emma* (Fig. 2; RBS = 89, PP = 0.95). Based on the K2P genetic distance estimation using 16S rRNA gene fragment (Table 2), the intraspecific genetic distances within *C. irawadi* range from 0.0% (between MZMU2385 and MZMU2596 from Mizoram) to 2.0% (between MZMU2230 from Mizoram and CESL 1089 from Arunachal Pradesh). Moreover, the genetic distance between the samples from Manipur (India) and the type locality in Sagaing (Myanmar) was only 1.5%, while 3.9% average interspecific distance was calculated for *C. irawadi* from Manipur and *C. versicolor* from Kerala + Tamil Nadu (India). According to Gowande et al. (2021), the intraspecific distance for 16S among the *C. irawadi* specimens was calculated to be up to 2.4%.

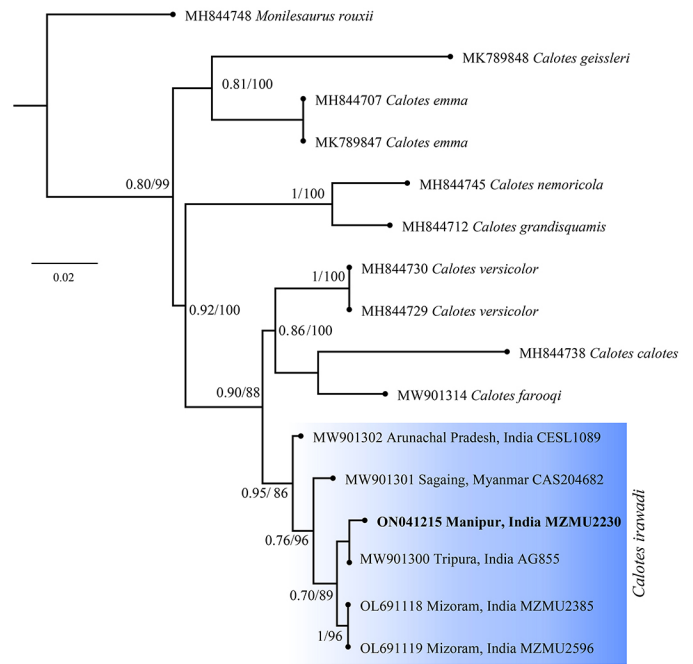


Fig. 2. Bayesian Inference (BI) tree based on mitochondrial 16S rRNA partial sequences. Numbers before slashes indicate Bayesian posterior probabilities (PP) and numbers after slashes indicate rapid bootstrap support (RBS) from the Maximum Likelihood phylogeny. The bolded individual is the sequence generated for this study.

In this work, we confirmed the occurrence of *C. irawadi* in Manipur using both morphological and molecular evidence.

Table 2. The K2P genetic divergence estimates among *Calotes* species using 16S rRNA sequences.

Sl. No.	NCBI Accession / Species	K2p distance														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	ON041215 <i>Calotes irawadi</i>															
2	MW901300 <i>Calotes irawadi</i>	0.004														
3	MW901301 <i>Calotes irawadi</i>	0.015	0.015													
4	MW901302 <i>Calotes irawadi</i>	0.020	0.015	0.013												
5	OL691119 <i>Calotes irawadi</i>	0.011	0.007	0.013	0.018											
6	OL691118 <i>Calotes irawadi</i>	0.011	0.007	0.013	0.018	0.000										
7	MH844738 <i>Calotes calotes</i>	0.066	0.066	0.066	0.066	0.072	0.072									
8	MK789847 <i>Calotes emma</i>	0.059	0.055	0.055	0.055	0.059	0.059	0.079								
9	MH844707 <i>Calotes emma</i>	0.059	0.055	0.055	0.055	0.059	0.059	0.079	0.000							
10	MW901314 <i>Calotes farooqi</i>	0.046	0.046	0.044	0.039	0.048	0.048	0.057	0.070	0.070						
11	MH844712 <i>C. grandisquamis</i>	0.070	0.068	0.072	0.070	0.068	0.068	0.094	0.070	0.070	0.088					
12	MK789848 <i>C. geissleri</i>	0.088	0.083	0.083	0.083	0.083	0.083	0.098	0.072	0.072	0.096	0.085				
13	MH844745 <i>C. nemoricola</i>	0.070	0.068	0.072	0.063	0.072	0.072	0.103	0.077	0.077	0.090	0.033	0.098			
14	MH844730 <i>C. versicolor</i>	0.039	0.035	0.042	0.033	0.042	0.042	0.063	0.059	0.059	0.046	0.079	0.092	0.072		
15	MH844729 <i>C. versicolor</i>	0.039	0.035	0.042	0.033	0.042	0.042	0.063	0.059	0.059	0.046	0.079	0.092	0.072	0.000	
16	MH844748 <i>Monilesaurus rouxii</i>	0.081	0.077	0.081	0.077	0.077	0.077	0.092	0.074	0.074	0.088	0.083	0.088	0.096	0.077	0.077

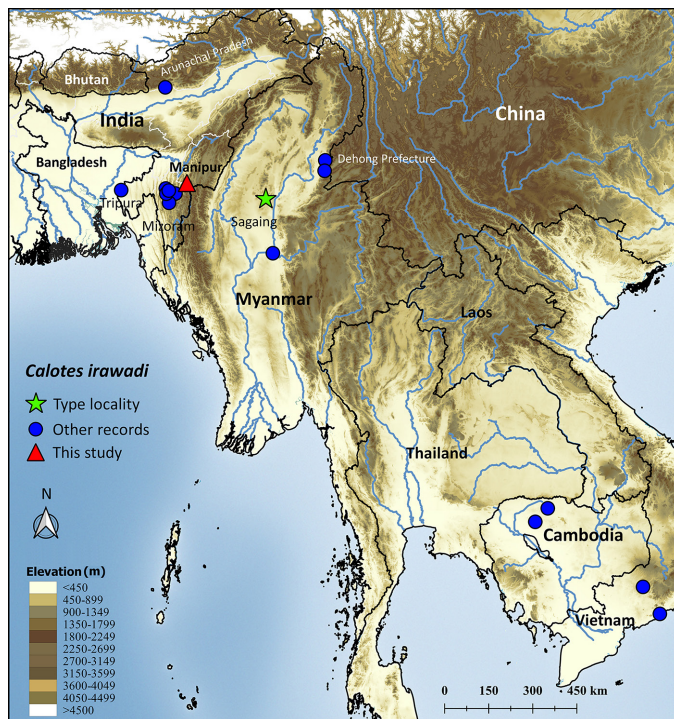


Fig. 3. Map showing distributional records of *Calotes irawadi*: the green star indicates the type locality (Zug et al. 2006), previous records are represented by blue circles (Gowande et al. 2021; Liu et al. 2021; Tariang et al. 2022), and the new record from this study is indicated by the red triangle.

The aerial distance from the present record at Sinzawl village, Manipur to the nearest locality at Tamdil National Wetland, Mizoram (Tariang et al. 2022) is approximately 51 km to the southwest (Fig. 3). Given the occurrence records of *C. versicolor* sensu stricto and *C. irawadi* (fide Gowande et al. 2021); it seems plausible that the earlier reports of *C. versicolor* in Manipur can also be referred to *C. irawadi*. In conclusion, we advocate generating more genetic information to address remaining taxonomic uncertainty for *Calotes* in northeastern India.

Acknowledgement

We acknowledge Chief Wildlife Warden of Environment, Forest Department, Government of Manipur for the herpetofaunal collection permit within Manipur State (permit no. (#3/22/2018-WL [Vol-II]). We are grateful to DST-SERB, New Delhi (DST No: EMR/2016/002391); DBT, New Delhi (DBT-NER/AAB/64/2017); DST-SERB, New Delhi (DST No. EEQ/2021/000243); DRDO, New Delhi (DGTM/DFTM/GIA/19-20/0422); International Herpetological Symposium, Inc. (IHS), USA; and The Rufford Foundation, UK (Grant No. 36737-1) for financial support. We appreciate Gospel Zothanmawia Hmar, Lalenzuala Tochwang and Lalhunthara Chawngthu for their field assistance.

Literature Cited

Darriba, D., D. Posada, A.M. Kozlov, A. Stamatakis, B. Morel, and T. Flouri. 2020. ModelTest-NG: a new and scalable tool for the selection of DNA and protein evolutionary models. *Molecular Biology and Evolution* 37: 291–4. <https://doi.org/10.1093/molbev/msz189>.

Decemson, Ht., M. Vabeiryureilai, L. Biakzuala, and H.T. Lalremsanga. 2021. Confirmation on the occurrence of *Calotes geissleri* Wagner, Ihlow, Hartman, Flecks, Schmitz and Böhme, 2021 (Sauria: Agamidae) in Manipur, India, with comments on its phylogenetic position. *Journal of Animal Diversity* 3: 14–19. <http://dx.doi.org/10.52547/JAD.2021.3.4.2>.

Edgar, R.C. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5: 1–19. <https://doi.org/10.1186/1471-2105-5-113>.

Eidler, D., J. Klein, A. Antonelli, and D. Silvestro. 2021. raxmlGUI 2.0: a graphical interface and toolkit for phylogenetic analyses using RAxML. *Methods in Ecology and Evolution* 12: 373–377. <https://doi.org/10.1111/2041-210X.13512>.

Giri, V.B., R. Chaitanya, S. Mahony, S. Lalrounga, C. Lalrinchhana, A. Das, V. Sarkar, P. Karanth, and V. Deepak. 2019. On the systematic status of the genus *Oriocalotes* Günther, 1864 (Squamata: Agamidae: Draconinae) with the description of a new species from Mizoram state, Northeast India. *Zootaxa* 4638: 451–484. <https://doi.org/10.11646/zootaxa.4638.4.1>.

Gowande, G., S. Pal, R. Masroor, P.U. Phansalkar, P. Dsouza, A. Jayarajan, and K. Shanker. 2021. Molecular phylogenetics and taxonomic reassessment of the widespread agamid *Calotes versicolor* (Daudin, 1802) (Squamata, Agamidae) across South Asia. *Vertebrate Zoology* 71: 669–696. <https://doi.org/10.3897/vz.71.e62787>.

Gray, J. 1846. Descriptions of some new species of Indian Lizards. *The Annals and Magazine of Natural History* 1: 429–430.

Kumar, S., G. Stecher, and K. Tamura. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33: 1870–1874. <https://doi.org/10.1093/molbev/msw054>.

Liu, S., C. Zuo, and D. Rao. 2021. Distribution extension of *Calotes irawadi* Zug, Brown, Schulte & Vindum, 2006, previously confused with *C. versicolor* (Daudin, 1802): first record from China. *Herpetozoa* 34: 83–88. <https://doi.org/10.3897/herpetozoa.32.e62596>.

Matthew, R. 2005. Reptilia, pp. 119–122. In: The Director (ed.), *Fauna of Manipur, State Fauna Series 10: (Part-1)*. Zoological Survey of India, Kolkata, India.

Pal, S., S.P. Vijayakumar, K. Shanker, A. Jayarajan, and V. Deepak. 2018. A systematic revision of *Calotes* Cuvier, 1817 (Squamata: Agamidae) from the Western Ghats adds two genera and reveals two new species. *Zootaxa* 4482: 401–450. <https://doi.org/10.11646/zootaxa.4482.3.1>.

Palumbi, S.R. (1996) Nucleic acids II: the polymerase chain reaction, pp. 205–247. In: D. Hillis, M. Moritz and B.K. Mable (eds.), *Molecular Systematics*. 2nd edition. Sinauer Associates Inc., Massachusetts, USA.

Rassmann, K. 1997. Evolutionary age of the *Galapagos iguanas* predates the age of the present Galapagos Islands. *Molecular Phylogenetics and Evolution* 7: 158–172. <https://doi.org/10.1006/MPEV.1996.0386>.

Ronquist, F. and J.P. Huelsenbeck. 2003. MrBayes3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574. <https://doi.org/10.1093/bioinformatics/btg180>.

Tariang, A.D., F. Malsawmdawngliana, L. Biakzuala, Ht. Decemson, L. Muansanga, L. Rinsanga, M. Vabeiryureilai, and H.T. Lalremsanga. 2022. Confirmation on the occurrence of *Calotes irawadi* in Mizoram, Northeast India. *Hamadryad* 39: 88–94.

Uetz, P., P. Freed, and J. Hošek (eds.). 2022. *The Reptile Database*. <<https://reptile-database.reptarium.cz/species?genus=Calotes&species=>

Wagner, P., F. Ihlow, T. Hartmann, M. Flecks, A. Schmitz, and W. Böhme. 2021. Integrative approach to resolve the *Calotes mystaceus* Duméril & Bibron, 1837 species complex (Squamata: Agamidae). *Bonn Zoological Bulletin* 70: 141–171. <https://doi.org/10.20363/BZB-2021.70.1.141>.

Zug, G.R., H.H.K. Brown, J.A. Schulte II, and J.V. Vindum. 2006 Systematics of the Garden Lizards, *Calotes versicolor* Group (Reptilia, Squamata, Agamidae), in Myanmar: Central Dry Zone Populations. *Proceedings of the California Academy of Science* 57: 35–68.