REPTILES & AMPHIBIANS

A New Distribution Record of the Kerala Skittering Frog, *Euphlyctis kerala* (Amphibia: Anura), from Kerala, India

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Dubois et al. (2021) separated the genus *Euphlyctis* Fitzinger 1843 into *Euphlyctis* and *Phrynoderma*. In 2022, Dufresnes et al (2022) made a taxonomic reassessment and placed all the species in one genus, *Euplyctis*. The Kerala Skittering Frog (*Euphlyctis kerala*) was recently described from The Thattekad Bird Sanctuary, Kerala, and has a restricted distribution south of the Palghat Gap (Dinesh et al. 2021). This frog is associated with freshwater ecosystems, especially ponds close to river tributaries (e.g., the Periyar River) at lower elevations (Frost 2021). Sister species include *E. aloysii* and *E. karaavali* from western coastal regions and *E. hexadactyla* from eastern coastal regions of India (Dinesh et al. 2022). A medium-sized frog, *E. kerala* is closely related and morphologically very similar to *E. aloysii*, but the ranges differ (Dinesh et al. 2021). The cryptic nature of frogs in this group makes field identification relatively difficult. The study conducted by Dinesh et al. (2021) presented grouping keys, making it easier to understand the distribution of this newly identified frog. *Euphlyctis kerala* was listed on the IUCN Red List as Data Deficient due to poor knowledge of their geographical distribution. In the present study, we used molecular tools to identify the distribution patterns of *E. kerala* from different parts of Kerala, India, by genetically identifying them with the help of mitochondrial 16S rRNA gene sequences. We also encountered and noted the distributions of *E. karaavali*, *E. hexadactyla*, *E. aloysii*, and *Euphlyctis cyanophlyctis* during this study.

As a part of amphibian surveys conducted in 2015–2019 in different parts of Kerala, we collected adult Kerala Skittering Frogs from four new locations (10.638 N, 76.567 E; 9.562 N, 76.351 E; 9.477 N, 76.582 E, and 10.742 N, 76.006 E), mostly from ponds during the early monsoon (June–July). We also collected specimens of *E. aloysii* and *E. karaavali* from Kerala and *E. hexadactyla* and *E. cyanophlyctis* from eastern coastal regions of Tamil Nadu for genetic comparisons (Fig. 1; Table 1). We took photographs of captured frogs and preserved toe clips in absolute alcohol for molecular work. After receiving antibiotic treatment, frogs were released at sites of capture. Morphological parameters followed Dinesh et al. (2021) and measurements were taken with Mitutoyo digital callipers to the nearest 0.1 mm.

In the field, *E. kerala* can be misidentified as the juveniles of *E. aloysii* or *E. karaavali*, all of which are in the *Hexadactylus*

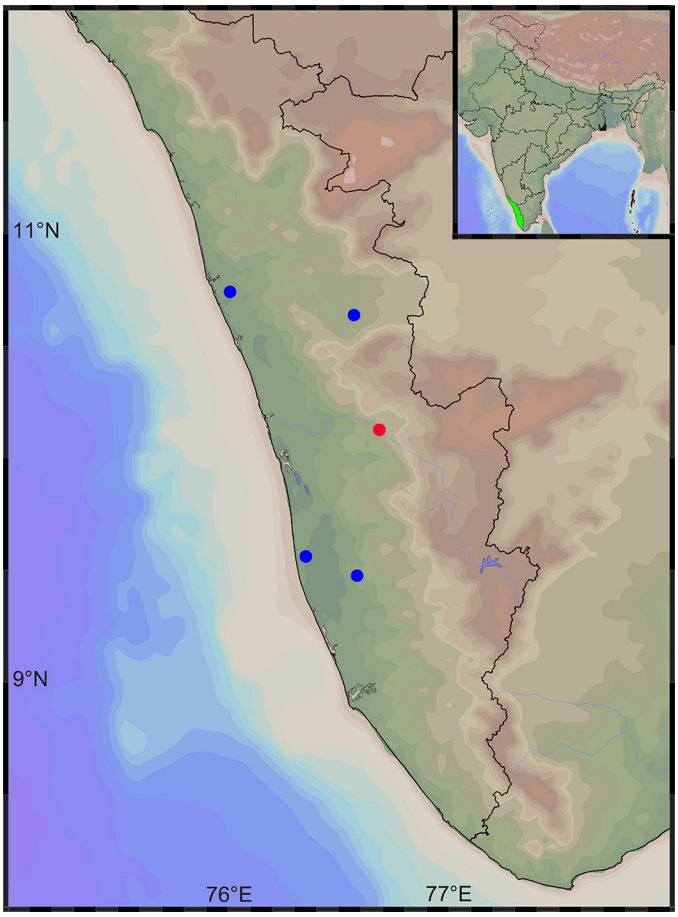

Figure 1. Map of southern India showing new collection localities of the Kerala Skittering Frog (*Euphlyctis kerala*) (blue dots) from Kerala and the type locality (red dot).

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Table 1. Species, localities, and NCBI accession numbers of sequences in the present study.

Sl. No.	Species Name	Location	16S GenBank accession number	Reference
1	*Euphlyctis kerala*	Pullode, Palakkad	ON005161 – ON005182	Present study
2	*Euphlyctis kerala*	Mannancherry, Alappuzha	ON013950 – ON013957	Present study
3	*Euphlyctis kerala*	Perumpanachi, Kottayam	ON013958, ON013959	Present study
4	*Euphlyctis kerala*	Changaramkulam, Malappuram	ON013960 – ON013962	Present study
5	*Euphlyctis kerala*	Western Costal Kerala	MH423737	Dinesh et al. 2021
6	*Euphlyctis kerala*	Western Costal Kerala	MH423738	Dinesh et al. 2021
7	*Euphlyctis kerala*	India	GU136102	Unpublished
8	*Euphlyctis aloysii*	Kozhikode	ON013932 – ON013938	Present study
9	*Euphlyctis aloysii*	Kannur	ON013939 – ON013941	Present study
10	*Euphlyctis aloysii*	Adayar	AB167939	Kurabayashi et al. 2005
11	*Euphlyctis karaavali*	Thiruvananthapuram	KU179083 – KU179087	Present study
12	*Euphlyctis karaavali*	Palakkad	ON005184 – ON005187	Present study
13	*Euphlyctis karaavali*	Uttara Kannada	KU870373	Priti et al. 2016
14	*Euphlyctis hexadactyla*	Puducherry	KU198183	Present study
15	*Euphlyctis hexadactyla*	Puducherry	ON016530 – ON016535	Present study
16	*Euphlyctis hexadactyla*	Puducherry	KU870376	Priti et al. 2016
17	*Euphlyctis cyanophlyctis*	Nagapattanam	KU179079	Present study
18	*Euphlyctis cyanophlyctis*	Puducherry	ON014040	Present study
19	*Euphlyctis cyanophlyctis*	Thiruvananthapuram	KU179080 – KU179082	Present study
20	*Euphlyctis cyanophlyctis*	Puducherry	MW872372	Dinesh et al. 2022
21	*Euphlyctis jaladhara*	Thattekad	MW872363	Dinesh et al. 2022
22	*Euphlyctis kalasgramensis*	Bangladesh	MG010388	Unpublished
23	*Euphlyctis ehrenbergii*	Yemen	AY014367	Kosuch et al. 2001
24	*Hoplobatrachus tigerinus*	Bangladesh	AB272591	Alam et al. 2008
25	*Hoplobatrachus tigerinus*	Bajipe	AB290412	Alam et al. 2008
26	*Hoplobatrachus tigerinus*	Padil	AB272594	Alam et al. 2008

clade (Dinesh et al. 2021). Males (SVL 28–31 mm) are usually smaller than females (SVL 34–38 mm), the dorsum bears a greenish inverted heart-shape between dorsolateral brown bands, the sides have white lines, eyes are shaped like diamonds, and the snout is pointed (Fig. 2). Measurements of the primarily male adults agree with data provided by Dinesh et al. (2021).

Genomic DNA was extracted from toe clips using a Qiagen DNeasy blood and tissue kit, and the 16S rRNA mitochondrial genes were amplified using forward (F-5'-CGCCTGTTTATCAAAAACAT-3') and reverse (R-5'-CCGGTCTGAACTCAGATCACGT-3') primers (Palumbi et al. 1991). A 10-µL PCR reaction mix was prepared containing 5.95 µL ddH_2O, 2 µL buffer (5X), 0.2 µL dNTPS (10 mM), 0.25 µL each primer (10 µM), 0.15 µL DMSO (100%), 0.2 µL Phire Hot start Taq polymerase (5 µg/µl), and 1 µL DNA template (10 µg/ml). The mixture was amplified at 98 °C of initial denaturation (30 s) followed by 30 cycles of denaturation at 98 °C (5 s), annealing at 58 °C (10 s), extension at 72 °C (10 s), followed by a final extension of 72 °C (1 m). PCR products were checked on a 2% agarose gel and purified using Exosap IT. DNA sequencing was performed using ABI PRISM® Big Dye Terminator v 3.1 Cycle Sequencing Kit in ABI PRISM 3730 capillary sequencer. Final sequences were submitted to GenBank (Table 1).

We used representative sequences from each locality and from the type locality and checked and corrected sequence ambiguities by aligning the sequences in Geneious Pro 6.0.5 (Kearse et al. 2012). The sequences were aligned using ClustalW built into BIOEDIT (Hall 1999; Thompson et al. 1994). Maximum Likelihood analysis was done by using 16S rRNA (515 bp) sequences using K2+G (Kimura2 parameter) as the best nucleotide substitution model in MEGA 7.0 software (Kumar et al. 2016). Branch evaluation was performed with 1,000 bootstrap replicates (Felsenstein 1985). Uncorrected *p*-values for 16S alignments were calculated using MEGA7 with a pairwise delete option.

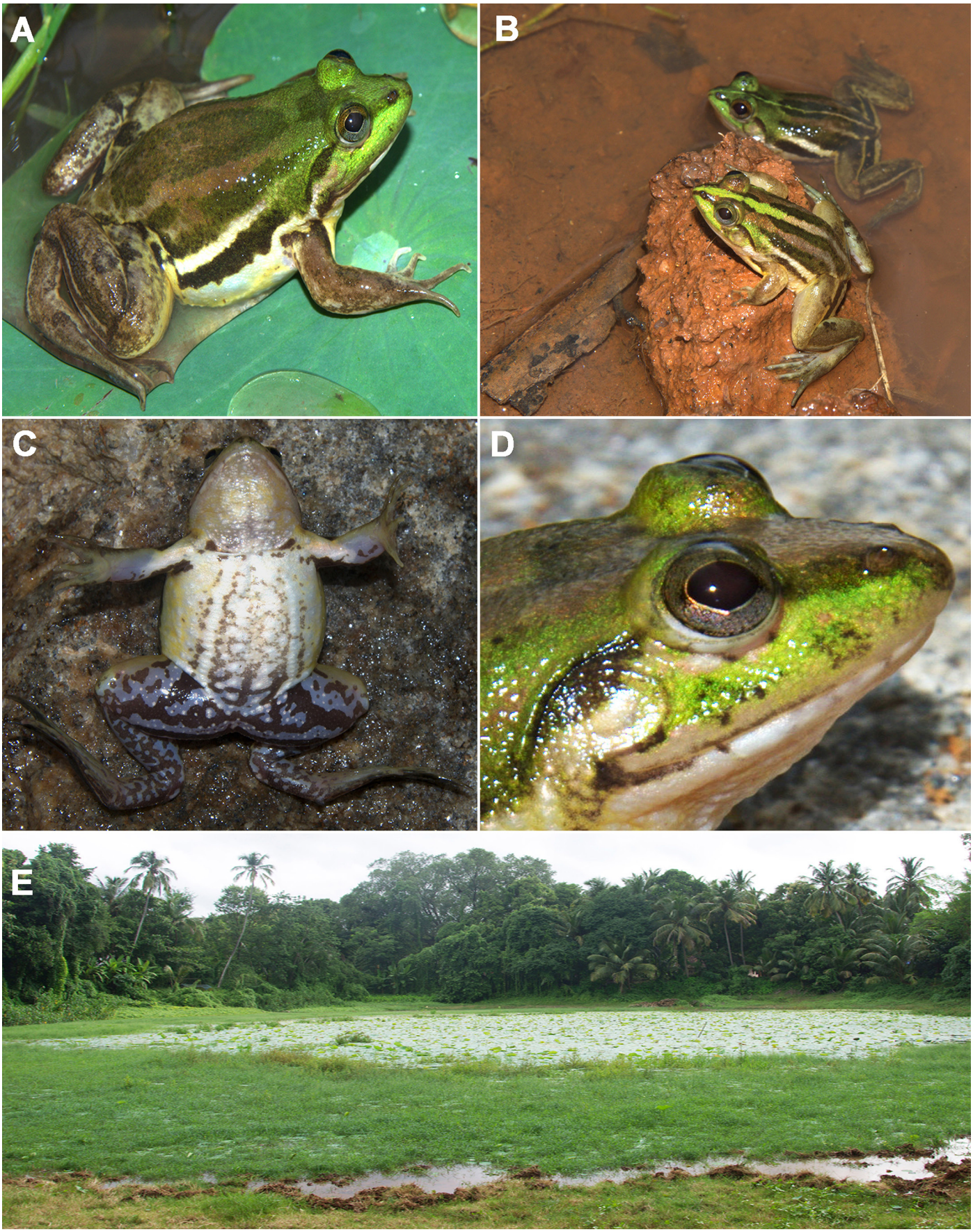

Figure 2. The Kerala Skittering Frog (*Euphlyctis kerala*) in its natural habitat (A & B), venter (C), head (D), and natural pond habitat (E). Photographs by Anoop V S.

We followed Frost (2021) for the current classification of *Euphlyctis* and used sequences of all species of *Euphlyctis* available in GenBank along with our material from the new localities. Phylogenetic analysis showed the clustering of *E. kerala* with the reference sample (MH423737) with high bootstrap values (Fig. 3). The cluster again confirms the existence of two separate lineages (Dinesh et al. 2022), first lineage consists of *E. kerala*, *E. aloysii*, *E. hexadactyla*,

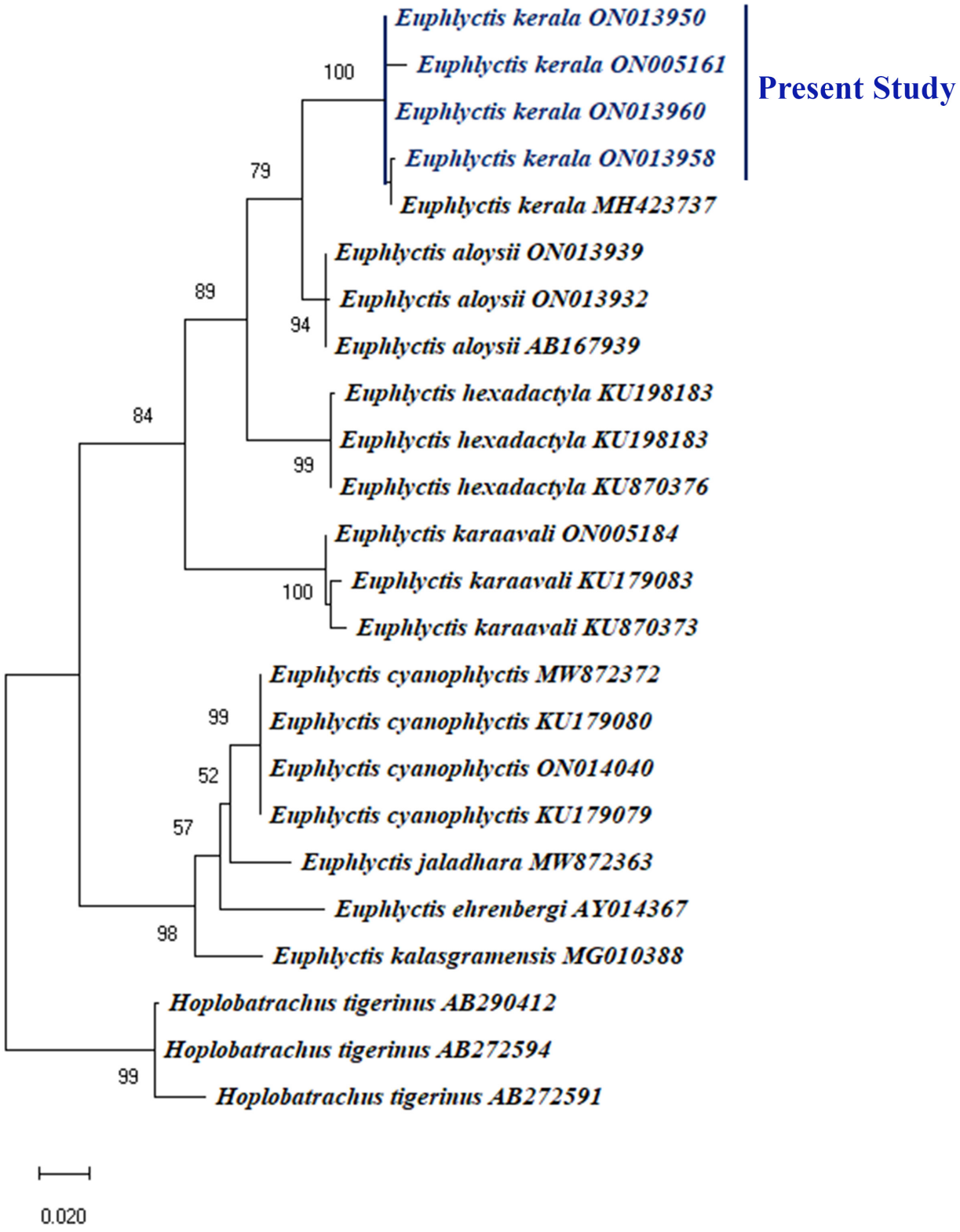

Figure 3. Maximum likelihood phylogenetic tree (Kimura2 parameter) of the Kerala Skittering Frog (*Euphlyctis kerala*) and its related species using 16S rRNA sequences in MEGA7. Numbers on nodes represent bootstrap support. Records in blue are from the present study.

and *E. karaavali* and the second consists of *E. jaladhara*, *E. cyanophlyctis*, *E. ehrenbergii*, and *E. kalasgramensis*. Pair-wise genetic distance varied ≤ 1% from the reference samples, confirming the identity of our frogs as *E. kerala* and had a genetic distance of 4.6% from the closest congener (*E. aloysii*) as in Dinesh et al. (2021) (Table 2). The discovery of *E. kerala* from these new regions extends the range of this species more than 100 km northward and 73 km to the south of the type locality and extends the range into lower elevations on either side of the Palghat Gap, Kerala.

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Table 2. Uncorrected pairwise genetic distances (%) between *Euphlyctis kerala* and congeners using 515 bp of mitochondrial 16S rRNA gene sequences.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	*Euphlyctis kerala ON005161*																					
2	*Euphlyctis kerala ON013950*	0.008																				
3	*Euphlyctis kerala ON013958*	0.012	0.004																			
4	*Euphlyctis kerala ON013960*	0.008	0.000	0.004																		
5	*Euphlyctis kerala MH423737*	0.010	0.002	0.002	0.002																	
6	*Euphlyctis aloysii ON013932*	0.046	0.038	0.042	0.038	0.040																
7	*Euphlyctis aloysii ON013939*	0.044	0.036	0.040	0.036	0.038	0.002															
8	*Euphlyctis aloysii AB167939*	0.044	0.036	0.040	0.036	0.038	0.002	0.000														
9	*Euphlyctis karaavali KU179083*	0.096	0.088	0.092	0.088	0.090	0.082	0.080	0.080													
10	*Euphlyctis karaavali ON005184*	0.096	0.088	0.092	0.088	0.090	0.078	0.076	0.076	0.006												
11	*Euphlyctis karaavali KU870373*	0.104	0.096	0.100	0.096	0.098	0.086	0.084	0.084	0.012	0.010											
12	*Euphlyctis hexadactyla KU198183*	0.066	0.058	0.062	0.058	0.060	0.054	0.052	0.052	0.082	0.080	0.088										
13	*Euphlyctis hexadactyla ON016530*	0.064	0.056	0.060	0.056	0.058	0.052	0.050	0.050	0.080	0.078	0.086	0.002									
14	*Euphlyctis hexadactyla KU870376*	0.064	0.056	0.060	0.056	0.058	0.052	0.050	0.050	0.080	0.078	0.086	0.002	0.000								
15	*Euphlyctis cyanophlyctis KU179079*	0.116	0.108	0.112	0.108	0.110	0.100	0.098	0.098	0.104	0.100	0.106	0.102	0.100	0.100							
16	*Euphlyctis cyanophlyctis ON014040*	0.116	0.108	0.112	0.108	0.110	0.100	0.098	0.098	0.104	0.100	0.106	0.102	0.100	0.100	0.000						
17	*Euphlyctis cyanophlyctis KU179080*	0.116	0.108	0.112	0.108	0.110	0.100	0.098	0.098	0.104	0.100	0.106	0.102	0.100	0.100	0.000	0.000					
18	*Euphlyctis cyanophlyctis MW872372*	0.116	0.108	0.112	0.108	0.110	0.100	0.098	0.098	0.104	0.100	0.106	0.102	0.100	0.100	0.000	0.000	0.000				
19	*Euphlyctis jaladhara MW872363*	0.124	0.116	0.120	0.116	0.118	0.108	0.106	0.106	0.102	0.098	0.104	0.104	0.102	0.102	0.032	0.032	0.032	0.032			
20	*Euphlyctis kalasgramensis MG010388*	0.110	0.102	0.106	0.102	0.104	0.106	0.104	0.104	0.104	0.100	0.106	0.102	0.100	0.100	0.042	0.042	0.042	0.042	0.046		
21	*Euphlyctis ehrenbergii AY014367*	0.132	0.124	0.128	0.124	0.126	0.114	0.112	0.112	0.118	0.116	0.122	0.110	0.108	0.108	0.046	0.046	0.046	0.046	0.054	0.062	

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