Alytes, 2009, **26** (1-4): 97-116.

Two new species of the genus *Euphlyctis* (Anura, Ranidae) from southwestern India, revealed by molecular and morphological comparisons

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Two new frog species of the genus *Euphlyctis*, which were shown to be two distinct taxa by mitochondrial DNA analyses, are described from Karnataka State, southwestern India. On the molecular phylogenetic tree, the first new species appears as a sister group with respect to *E. hexadactylus*. The second new species forms a group with *E. cyanophlyctis*. The first species differs from *E. hexadactylus* in having a distinctly smaller snout-vent length and dark brown bold markings on the dorsum, a smaller head, shorter hindlimbs and wider eyelids, relative to snout-vent length. The second species differs from the close relative *E. cyanophlyctis* in having shorter fingers. Its advertisement calls are composed of trills that are much longer in duration, are composed of more numerous pulses, and have a lower dominant frequency than those of *E. cyanophlyctis* and *E. hexadactylus*. Morphological comparisons between the four species are presented. The present study reveals hitherto overlooked cryptic biodiversity in the genus *Euphlyctis*.

INTRODUCTION

Euphlyctis is a small genus comprising only four currently recognized species: *E. cyanophlyctis* (Schneider, 1799) from Iran, Afghanistan, Pakistan, Nepal, India, Sri Lanka, Malaya and Vietnam; *E. ehrenbergii* (Peters, 1863) from Saudi Arabia and Yemen; *E. ghoshi* (Chanda, 1991) from Manipur, India; and *E. hexadactylus* (Lesson, 1834) from India, Sri Lanka and Bangladesh (FROST, 1985; CHANDA, 1991; DUBOIS, 1992). *Euphlyctis cyanophlyctis* and *E. hexadactylus* are known to occur in southwestern India (BIJU, 2001; DANIELS, 2005). These species are aquatic or semi-aquatic frogs with wide toe webbing that usually live half-submerged in water, or on the water edge of ponds, wetlands, paddy fields and ditches.

In 2003, we collected small frogs of the genus *Euphlyctis* from Mangalore, together with *E. hexadactylus* and *E. cyanophlyctis*. At first, we considered the small ones as juveniles of *E. hexadactylus*. However, mtDNA data revealed that the small frogs were distinctly different from *E. hexadactylus* as well as from *E. cyanophlyctis* (KURABAYASHI et al., 2005; ALAM et al., 2008). We collected similar small *Euphlyctis* frogs from Mudigere in the Western Ghats in 2007, and the mtDNA data, described in the present study, clarified that the frogs from Mudigere differed from those of Mangalore. ALAM et al. (2008) also demonstrated the presence of another cryptic *Euphlyctis* species from Bangladesh by mtDNA analysis, but the two new Indian taxa here treated were clearly different from that from Bangladesh. These latter two Indian frogs are described below as two new species.

Recently, many new anuran species have been described from southwestern India, including the Western Ghats (e.g., DUBOIS et al., 2001; BIJU & BOSSUYT, 2003, 2005, 2006; KURAMOTO & JOSHY, 2003; BIJU et al., 2007; KURAMOTO et al., 2007). This indicates that the wealth of amphibian biodiversity in this area is beyond the expectation generally recognized. The present study and other recently obtained evidence sheds light on the cryptic biodiversity in the small and rather unnoticed genus *Euphlyctis*.

MATERIAL AND METHODS

Euphlyctis frogs were collected from Adyar (12°52'N, 74°55'E; altitude 1 m) and Bajpe (12°58'N, 74°50'E; altitude ca. 70 m) in Mangalore, Dakshin Kannad District of Karnataka, and from Mudigere (13°07'N, 75°31'E; altitude ca. 1020 m), Chikumagalur District of Karnataka, during the rainy season (May to July), from 2003 to 2008. To elucidate the genetic divergence and phylogenetic relationship of the *Euphlyctis* taxa occurring in southwestern Karnataka, partial mtDNA portions corresponding to 12S and 16S rRNA genes were analyzed for 37 *Euphlyctis* samples involving those of *E. hexadactylus* from Adyar and *E. cyanophlyctis* from Bajpe, Padil (Mangalore), Karnoor (Dakshin Kannad District) and Madikeri (Kodagu District).

In the present study, the mtDNA fragments were newly amplified and sequenced for 14 specimens and the data of the remaining 23 taxa were obtained from our previous studies (ALAM et al., 2008). The DNA amplification and sequence strategies followed the procedures as in the previous papers. The resultant sequences of each 12S and 16S rRNA gene were initially aligned using ClustalX 1.83 (THOMPSON et al., 1997); the initial 12S and 16S rRNA alignment data contained 566 and 520 nucleotide sites, respectively. From these alignment data, the genetic divergence (uncollected p value) between taxa was calculated. To perform sophisticated phylogenetic analyses, gaps and ambiguous alignment sites were excluded from the initial alignments using Gblocks 0.91b (CASTRESANA, 2000). To check whether 12S and 16S rRNA data could be submitted to combined analyses, a permutation homology test (FARRIS et al., 1995) was conducted using PAUP* 4.10b (SWOFFORD, 2001) (P = 0.124). Then, the two gene data were concatenated. The concatenated alignment data contained a total of 976 nucleotide sites, 192 of which were parsimoniously informative. Phylogenetic analyses based on the concatenated data were conducted

using maximum likelihood (ML) and Bayesian inference (BI) methods. In these analyses, *Fejervarya limnocharis* (accession no. AY158705; LIU et al., 2005) and *Limnonectes fujianensis* (AY974191; NIE et al., unpublished) were used as outgroups. For ML and BI analyses, appropriate substitution models were estimated using Akaike information criteria implemented in Modeltest 3.7 (POSADA & CRANDALL, 1998), and a general time-reversible substitution model with gamma population and proportion of invariable sites sub-models (GTR+G+I) was chosen. ML analysis was performed using PAUP*. Nonparametric bootstrap (BP) values under ML were calculated with 300 replicates. BI analysis was performed using MrBayes 3.1.2 (RONQUIST & HUELSENBECK, 2003). The following settings were also used for the BI analysis: number of Markov chain Monte Carlo generations = 15×10^5 and sampling frequency = 10. The burn-in size was determined by checking convergences of –log likelihood ($-\ln L$) values, and the first 1×10^5 generations were discarded. The statistical support of the resultant BI tree was evaluated by Bayesian posterior probabilities (BPP).

Measurements were recorded for snout-vent length (SVL), head length (HL), head width (HW), snout to nostril distance (S-N), inter-nostril distance (N-N), nostril to eye distance (N-E), eye diameter (ED), inter-orbital distance (E-E), eyelid width (ELW), tympanum diameter (TD), hand length (HAL), no. 1 to no. 4 finger length (F1-F4), hindlimb length (HLL), femur length (FEL), tibia length (TIL), foot length (FOL), and no. 1 to no. 5 toe length (T1-T5). For details of the method of measurements see KURAMOTO & JOSHY (2006) and KURAMOTO et al. (2007). Juvenile specimens were excluded from measurements. For morphological comparison, we measured six preserved specimens of *E. hexadactylus* from Adyar, Mangalore and 19 specimens of *E. cyanophlyctis* from Mangalore, Karnoor, Bhatkal, Talagini, Mudigere and Madikeri, all in Karnataka State (see fig. 1 in KURAMOTO et al., 2007), deposited in the Rondano Biodiversity Research Laboratory, St. Aloysius College. Examined specimens are listed below except for those of the new species. Discriminant analyses were performed by SPSS (15.0J) statistics software (SPSS Japan, Inc.) using the measurements without any transformation.

Euphlyctis cyanophlyctis. – Bajpe: RBRL 04070611, 05072202, 07072114 (1 adult ♂, 2 adult ♀). Bhatkal: RBRL 00062601-00062603, 00062605-00062607 (6 adult ♀). Karnoor: RBRL 01080508, 04071139, 04071140 (2 adult ♂, 1 adult ♀). Madikeri: RBRL 03060702 (1 adult ♀). Mudigere: RBRL 05070921, 05070922 (1 adult ♂, 1 adult ♀). Padil: RBRL 03052303 (1 adult ♀). Talagini: RBRL 01081113, 01081114, 01081118 (3 adult ♀).

Euphlyctis hexadactylus. – Adyar: RBRL 03060601, 05071901-05071903, 07072801, 07072802 (5 adult ♂, 1 adult ♀).

The advertisement calls were recorded in Mudigere on 29 July 2007 at an air temperature of 23.2°C and on 27 July 2008 at 21.0°C using an MD recorder (Sony MZ-B10). The recorded calls were analyzed by Avisoft-SASLab Light software (Avisoft Bioacoustics).

The type specimens were deposited in the Natural History Collections of the Bombay Natural History Society (BNHS), and the other specimens were stored in the Rondano Biodiversity Research Laboratory, St. Aloysius College (RBRL).

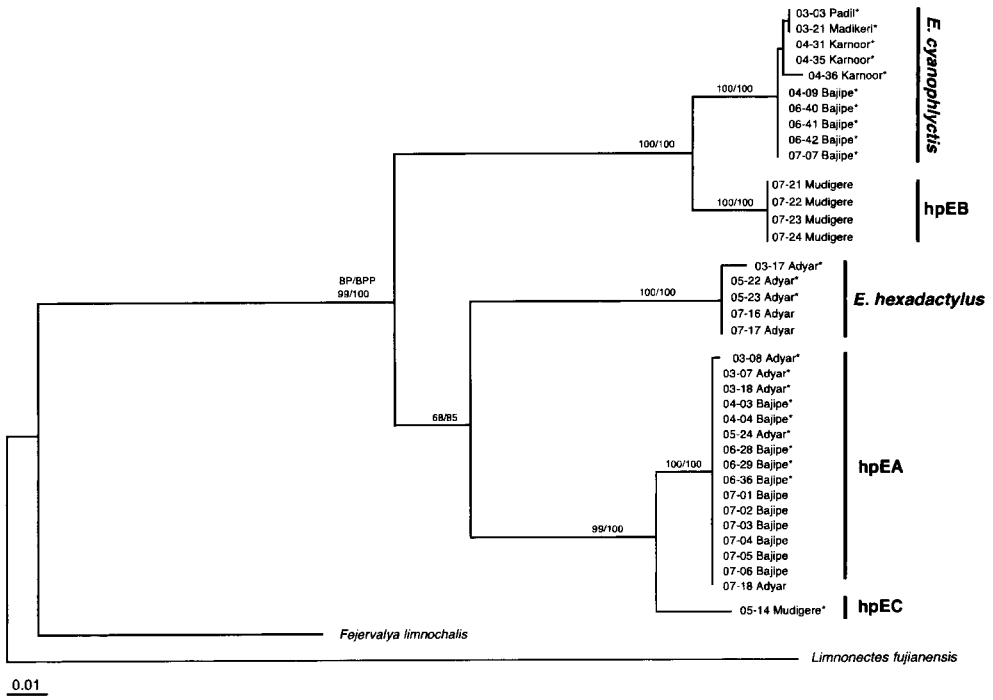

Fig. 1. – Phylogenetic relationships of *Euphlyctis* taxa from Karnataka, India, inferred from mitochondrial 12S and 16S rRNA gene data. Maximum likelihood tree ($-\ln L = 3356.93$) is represented here. Bayesian analysis reconstructed the same tree topology. The numbers on the nodes are BP in ML and BPP in BI. Three haplotype groups are shown by abbreviations, hpEA, hpEB and hpEC. Field numbers of samples and collecting sites are shown. Asterisks indicate that the samples were used in analyses by KURABAYASHI et al. (2005) and ALAM et al. (2008).

RESULTS

MOLECULAR PHYLOGENY AND GENETIC DIVERGENCE OF THE *EUPHLYCTIS* TAXA FROM KARNATAKA

Based on the 12S and 16S rRNA gene sequences, the Indian *Euphlyctis* specimens consisted of five major haplotype groups (fig. 1). Two of the five groups corresponded to *E. cyanophlyctis* and *E. hexadactylus*, and the others were temporarily named as hpEA, hpEB and hpEC. In the ML tree (fig. 1), the hpEB group formed a group with *E. cyanophlyctis* and this clade was strongly supported by statistical values (BP = 100; BPP = 100). The hpEA and hpEC groups formed a group, and they became a sister taxon with respect to *E. hexadactylus*, but statistical support for this relationship was not high (BP = 68; BPP = 85). The same relationships as for the five major *Euphlyctis* taxa were also reconstructed in our Bayesian analysis. Furthermore, the present result was partially congruent with the results of previous studies. KURABAYASHI et al. (2005) showed that small-sized *Euphlyctis* specimens (hpEA)

from Mangalore (Adyar and Bajpe) differed genetically from *E. hexadactylus,* and ALAM et al. (2008) found that one specimen from Mudigere (hpEC) was closely related to the hpEA group, but there was a degree of genetic divergence between the groups.

According to ALAM et al. (2008), the average sequence divergences between *E. hexadactylus* and hpEA (Ehex-In1 and Ehex-In2 in ALAM et al., 2008) were 11.9 % and 6.3 % for 12S and 16S rRNA genes, respectively. Because these values were larger than those previously reported from intraspecific sequence comparisons in mantellids (VENCES et al., 2005) and South American bufonids and hylids (FOUQUET et al., 2007), ALAM et al. (2008) concluded that the two haplotype groups should be separated taxonomically as different species. When we recalculated the average sequence divergence between these taxa with the present additional material, the values were 13.0 % and 9.1 % for 12S and 16S rRNA genes, respectively. The specimen from Mudigere collected in 2003 (hpEC; Ehex-In3 in ALAM et al., 2008) was also separated clearly from *E. hexadactylus* (15.3 % and 9.1 % for 12S and 16S), but the sequence divergence values (5.0 % and 2.3 %) did not support the distinct separation between the hpEC and hpEA groups. Only one specimen with the hpEC haplotype has been found so far, and this specimen was apparently subadult. Thus, more specimens are needed before discussing its taxonomic status.

The most remarkable finding in the present study was that the five specimens from Mudigere (hpEB) collected in 2007 formed a sister group to that of *E. cyanophlyctis* (fig. 1). Molecular divergence between hpEB and *E. cyanophlyctis* was 16.4 % for 12S and 10.7 % for 16S rRNA genes. As in the case between hpEA and *E. hexadactylus*, these values were large enough to regard the hpEB group as a distinct species from *E. cyanophlyctis*.

Our molecular analyses have revealed the occurrence of two undescribed species in southwestern part of Karnataka. As discussed in the later section, the two haplotype (hpEA and hpEB) groups were morphologically distinct from *E. hexadactylus* and *E. cyanophlyctis,* respectively, and from each other. These indicate that the two haplotype groups are reproductively distinct, and are described below as new species.

TAXONOMY

Euphlyctis aloysii sp. nov.
(fig. 2-3)

hpEA group in fig. 1 and in KURABAYASHI et al. (2005).
Ehex-In2 group in ALAM et al. (2008).

Diagnosis. – Small *Euphlyctis* species, SVL from 31.8 to 45.2 mm in females. It differs from *E. hexadactylus* in its distinctly smaller body size, having four large elliptical dark markings on the dorsum, smaller head, shorter hindlimbs, and wider eyelids, relative to SVL. The presence of large dorsal markings and thin mid-dorsal stripe readily distinguishes this species from *E. cyanophlyctis.* The eyes and tympanums are smaller, and femur and tibia are shorter, relative to SVL, in *E. aloysii* than in *E. cyanophlyctis.*