

# New insights into the taxonomy of the skittering frog *Euphlyctis cyanophlyctis* complex (Schneider, 1799) (Amphibia: Dicroglossidae) based on mitochondrial 16S rRNA gene sequences in southern Asia

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**Abstract.** The Skittering frog (*Euphlyctis cyanophlyctis*) is considered to be a species complex distributed in southern and southeastern Asia. Genetic diversity and taxonomic status of populations across their ranges is unclear and existence of several cryptic species is expected. In this study we used sequence variation in the mitochondrial 16S ribosomal RNA gene to elucidate the taxonomic status of Iranian populations of *E. cyanophlyctis* and compare their genetic diversity and divergence to populations from the Indian subcontinent. Phylogenetic analysis indicated that the populations of *E. cyanophlyctis* from Iran, Bangladesh-Assam (northeastern India), southern India, and Sri Lanka are partitioned into different clades. At least four different haplogroups were detected which are here proposed to be considered as allopatric cryptic species. The sedimentation of the Helmand River into the Sistan depression during the Neogene and subsequent formation of dry land barriers are proposed to have caused the Iranian populations of skittering frogs to be disconnected from those of the Indian subcontinent, resulting in differentiation of these lineages. In addition, some populations from southern India and those from Sri Lanka that were previously recognized as *E. cyanophlyctis* belong to *E. mudigere*. A preliminary investigation on the genetic diversity of the populations from southeastern Iran highlights the low genetic diversity among these populations.

**Keywords.** *Euphlyctis cyanophlyctis*, phylogeny, 16S rRNA gene, differentiation.

## INTRODUCTION

The genus *Euphlyctis* (family Dicroglossidae) consists of six species of which *Euphlyctis cyanophlyctis* (Schneider, 1799) is one of the most widespread in southern Asia. This species has been assigned to various genera over time, including *Rana* (*Rana cyanophlyctis* Boulenger, 1920), *Bufo* (*Bufo cyanophlyctis* Latreille, 1801), and *Dicroglossus* (*Dicroglossus cyanophlyctis* Deckert, 1938),

and it has been revised in its current name by Dubois (1975). The type locality of this species is probably Tranquebar in southeastern India (Bauer, 1998) and the species is believed to be distributed throughout southern Asia from southeastern Iran to Vietnam and Sri Lanka (Frost, 2013).

The distribution and taxonomy of Iranian populations of *E. cyanophlyctis* have been debated by several authors. Boulenger (1920) mentioned that Blanford

recorded it from Makran, Persian Baluchestan. Earlier, Nikolskii (1899) described *Rana cyanophlyctis seistanica* from Sistan, Iran based on its smaller tympanum and longer snout. This subspecies has only been reported from the type locality and along the border of Iran, Pakistan, Afghanistan (Khan, 2004). Anderson (1963) later recorded *Rana cyanophlyctis* from Iran. Baluch and Kami (1995) reported *Rana cyanophlyctis cyanophlyctis* from southern and southeastern Iran and proposed *Rana cyanophlyctis seistanica* to be a synonym. Additionally, Khan (1997) described a new subspecies, *Euphlyctis cyanophlyctis microspinulata* from Khuzdar, Baluchestan, Pakistan and recognized three subspecies in his checklist of the amphibians of Pakistan (Khan, 2004).

The previous phylogenetic analysis by Kosuch et al. (2001) based on a fragment of the mitochondrial 16S ribosomal RNA gene suggested a close relationship between *E. cyanophlyctis* and *E. ehrenbergii*. A subsequent analysis suggested a sister relationship between *E. cyanophlyctis* and *E. hexadactylus* (Kurabayashi et al., 2005). Alam et al. (2008) studied the genetic divergence of two species in this genus, *E. cyanophlyctis* and *E. hexadactylus*, and recognized at least two cryptic species among the populations of *E. cyanophlyctis* from India, Sri Lanka and Bangladesh. In addition, a new species, *Euphlyctis mudigere*, was described recently from southwestern India (Joshy et al., 2009).

During the late Paleocene and early Eocene, the Indian plate, which had broken away from Gondwana in the Early Cretaceous, collided into the Eurasian landmass and formed the Sistan depression along its western part on the Iranian microcontinent. Later, during the Neogene, the Sistan depression was filled as a result of continuous sedimentation of Helmand River (Molnar and Tapponnier, 1975; Klootwijk and Pierce, 1979). The region is today a transition between the Oriental and Palaearctic faunas and contains a mixture of elements of both (Misonne, 1959; Borkin and Litvinchuk, 2012). However, the Helmand Basin serves as a low altitude barrier (Macey et al., 1998; Rastegar Pouyani et al., 2010) between Iranian Plateau and Indian subcontinent.

This area in southeastern Iran comprises dry lands irrigated by the Helmand River and includes Hamoon Lake (Whitney, 2006). The region also comprises a part of the Lut Desert, one of the most arid deserts in the world extending to the Dashti Margo (desert of death) in Afghanistan. Low precipitation and a large excursion between day and nighttime temperatures leads to a low diversity of amphibian in this region except for infrequent aquatic habitats associated with the Helmand River that provide suitable habitats for populations of *E. cyanophlyctis*.

In this study, we address the taxonomic status of different populations of *E. cyanophlyctis* from Sistan, south-eastern Iran. We analyzed intraspecific variation within different populations of *E. cyanophlyctis* of southern Asia and also investigated the phylogenetic relationships among populations of five species of the genus *Euphlyctis* in southern Asia to assess the taxonomic status of the species complex.

## MATERIALS AND METHODS

### Specimens

A total of 41 individuals of *E. cyanophlyctis* were collected using hand nets in 2010 to 2011 from nine localities in Sistan and Baluchestan province in southeastern Iran (Fig. 1). Identification of specimens was based on Baluch and Kami (1995). All specimens are deposited in the Zoological Laboratory of Faculty of Agriculture and Natural Resource, Department of Crop Productions Technology, Saravan, Iran. Tissue samples were available from 32 specimens of the 41 specimens of *E. cyanophlyctis* as well as two *Pseudepidalea viridis* (family Bufonidae) from Iran that were used as outgroups (Table 1 and Fig. 1). All tissue samples were taken from the musculature of the hind limbs and preserved in 80% ethanol.

### Laboratory procedures

Total genomic DNA was extracted from ethanol-preserved muscle using a standard phenol/chloroform protocol (Sambrook et al., 2001). Standard polymerase chain reaction (PCR) amplifications were performed to amplify 492 bp of the mitochondrial 16S ribosomal RNA gene fragment (hereafter 16S). PCR amplification was performed using the universal forward primer 16SL (5'-CGCCTGTTTATCAAAAACAT-3') and the reverse primer 16SH (5'-CCGGTCTGAACTCAGATCACG-3') following the protocol of Palumbi (1991).

The amplified DNA fragments were sequenced commercially using the automated sequencer ABI prism 3700 at Macrogen Inc. (South Korea). Newly obtained sequences were deposited in GenBank (KF992800 to KF992833) (Table 1). In addition to specimens from Iran, 21 additional 16S sequences of the genus *Euphlyctis* and one of the dicroglossid *Hoplobatrachus tigerinus* were obtained from GenBank (Table 1).

### Phylogenetic analyses

Nucleotide sequences were aligned using ClustalW (Thompson et al., 1994) as implemented in the Bioedit sequence alignment editor, v.7.0.9 (Hall, 1999). Gaps in the alignment were treated as missing data. Genetic distances were calculated with MEGA v.4.0 (Tamura et al., 2007) with the p-distances model using the complete deletion option (Kimura, 1980). Phylogenetic reconstructions were carried out under the maximum

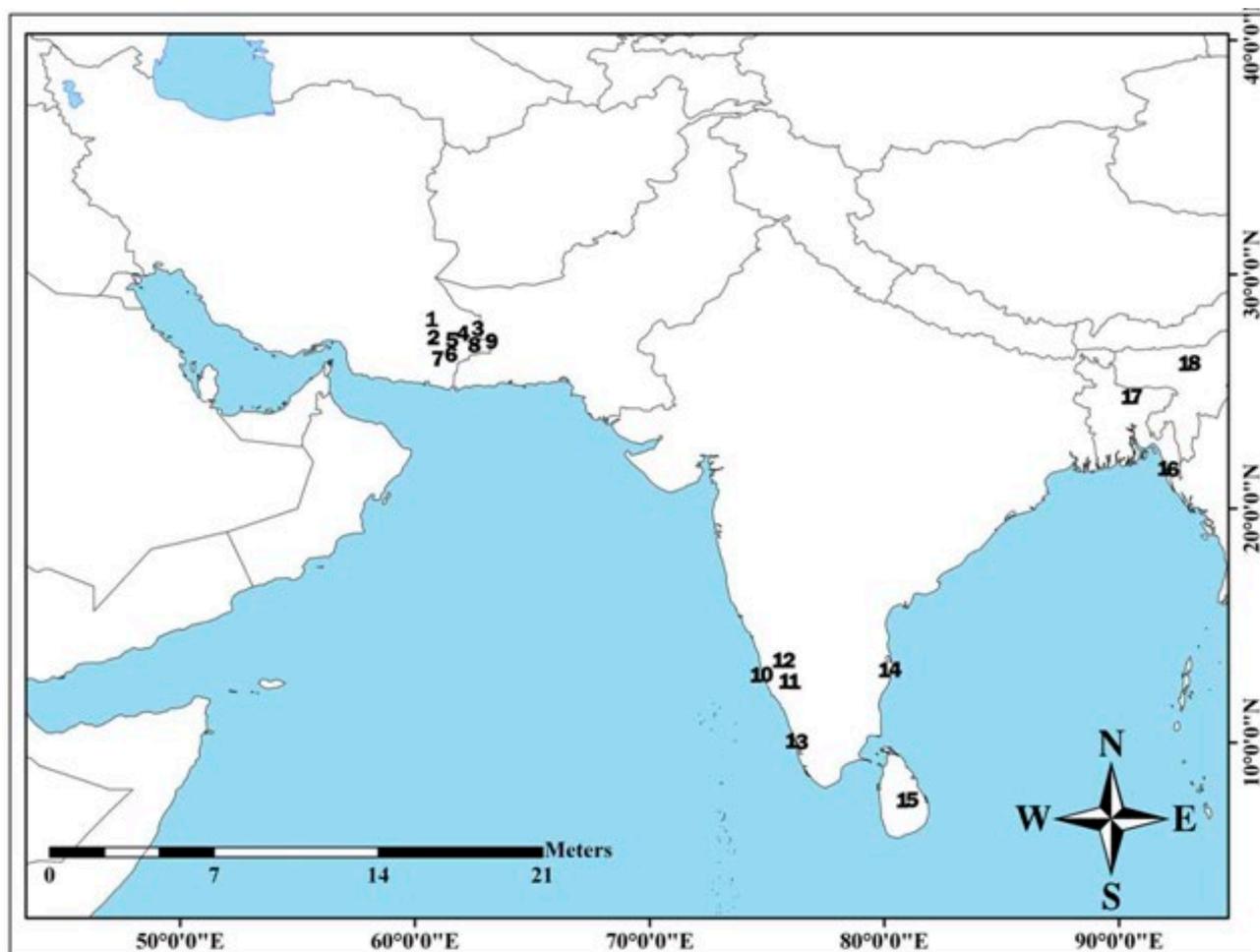


Fig. 1. Map showing the sampling localities of the *Euphlyctis cyanophlyctis* used for this study.

parsimony (MP) and the maximum likelihood (ML) methods in PAUP\* v.4.0b10 (Swofford, 2000). Appropriate substitution models were determined using the Akaike information criterion, as implemented in Modeltest (Posada and Crandall, 1998).

Maximum likelihood analyses were carried out using a heuristic tree search with 10 random addition sequence replicates and tree-bisection-reconnection (TBR) branch swapping. Maximum parsimony analyses were performed using heuristic searches with TBR branch swapping and random addition sequence with 1000 replicates. Support for internal nodes was estimated via nonparametric bootstrapping (500 and 5000 replicates for ML and MP, respectively) (Felsenstein, 1985) with a single random addition sequence replicate per bootstrap replicate.

Bayesian inference (BI) reconstructions were performed using MrBayes v.3.2.2 (Huelsenbeck et al., 2011). Four simultaneous Markov chain Monte Carlo (MCMC) chains with incremental heating temperature 0.2 were run 6000,000 generations and sampled every 100 generations. The burn-in size was determined by checking the convergence of  $-\log$  likelihood ( $-\ln L$ ) values, and the first 10% of the MCMC chain was discarded. The Bayesian posterior probability (PP) was evaluated from the

remaining trees. Haplotype and nucleotide diversity indexes for evaluation of DNA polymorphism were estimated using DnaSP v.5 (Librado and Rozas, 2009). The parsimony network of genealogical relationships within *E. cyanophlyctis* was obtained TCS v.1.21 (Clement et al., 2000).

## RESULTS

Phylogenetic analyses were conducted on a final alignment of 492 bp of 16S for 53 individuals of *Euphlyctis*, as well as two *P. viridis* and one *H. tigerinus* that served as outgroups. The alignment contained 81 variable sites, of which 48 were parsimony informative. Gaps were coded as missing and there were no ambiguous sites. The best-fit model obtained from Modeltest was a general time-reversible substitution model (GTR+I+G) ( $-\ln L=1782.6420$ ), with a proportion of invariable sites  $I=0.3651$ , variable sites  $G=0.4488$ , empirical base frequencies (A: 0.3035; C: 0.2413; G: 0.2079; T: 0.2473), and

**Table 1.** Tissue samples and GenBank accession numbers as well as locality information for the *Euphlyctis cyanophlyctis* specimens used in this study. Number of specimens (n), number of haplotype (H), haplotype diversity (Hd  $\pm$ SD) and nucleotide diversity ( $\pi \pm$ SD). Sequences not generated in the framework of this study were obtained from GenBank from following publications: a: Bossuyt and Milinkovitch (2000); b: Kosuch et al. (2001); c: Kurabayashi et al. (2005); d: Alam et al. (2008); e: Joshy et al. (2009); f: Kotaki et al. (2010); g: Hasan et al. (2012). Sequences with accession numbers starting with KF were generated for the present study.

Species	Collecting site		n	Locality number	H	Hd	$\pi$	Accession Number
	Country	Locality						
<i>E. cyanophlyctis</i>	Iran	Karvandar	3	1	2	0.353 $\pm$ 0.084	0.00072 $\pm$ 0.00017	KF992818-KF992820
	Iran	Irendeghan	3	2				KF992821-KF992823
	Iran	Jalgh	3	3				KF992809-KF992811
	Iran	Apatan	6	4				KF992800-KF992805
	Iran	Zaboli	3	5				KF992829-KF992831
	Iran	Ashar	2	6				KF992827 and KF992828
	Iran	Sarbaz	3	7				KF992824-KF992826
	Iran	Domaki	3	8				KF992806-KF992808
	Iran	Tiran	6	9				KF992812-KF992817
	India	Padil	2	10	2	0.567 $\pm$ 0.129	0.00262 $\pm$ 0.00066	AB272603-AB272604 <sup>d</sup>
	India	Mangalore	1	10				AB488901 <sup>f</sup>
	India	Konaje	1	11				JF832392
	India	Madikeri	1	11				AB167938 <sup>c</sup>
	India	Cochin	1	13				AY014366 <sup>b</sup>
	India	Assam	1	18	5	0.857 $\pm$ 0.108	0.00484 $\pm$ 0.00075	AB290420 <sup>d</sup>
	Bangladesh	Mymensingh	3	17				AB272602 <sup>d</sup> , AB530495 <sup>g</sup> , AB272601 <sup>d</sup>
	Bangladesh	Cox's Bazar	4	16				AB530494 <sup>g</sup> , AB530496-AB530498 <sup>g</sup>
<i>E. mudigere</i>	Sri Lanka		2	15	3	0.833 $\pm$ 0.222	0.00378 $\pm$ 0.00378	AB290418-AB290419 <sup>d</sup>
	India	Mudigere	2	12				AB377109 <sup>e</sup> , AF249053 <sup>a</sup>
<i>E. aloysii</i>	India	Adyar	1	14	-	-	-	AB272606 <sup>e</sup>
<i>E. ehrenbergii</i>	Yemen		1	-	-	-	-	AY014367 <sup>b</sup>
<i>E. hexadactylus</i>	India	Mangalore	1	10	-	-	-	AB167941 <sup>c</sup>
<i>H. tigerinus</i>	India	Padil	1	10	-	-	-	AB272594 <sup>d</sup>
<i>P. viridis</i>	Iran	Zabol	2	-	-	-	-	KF992832-KF992833

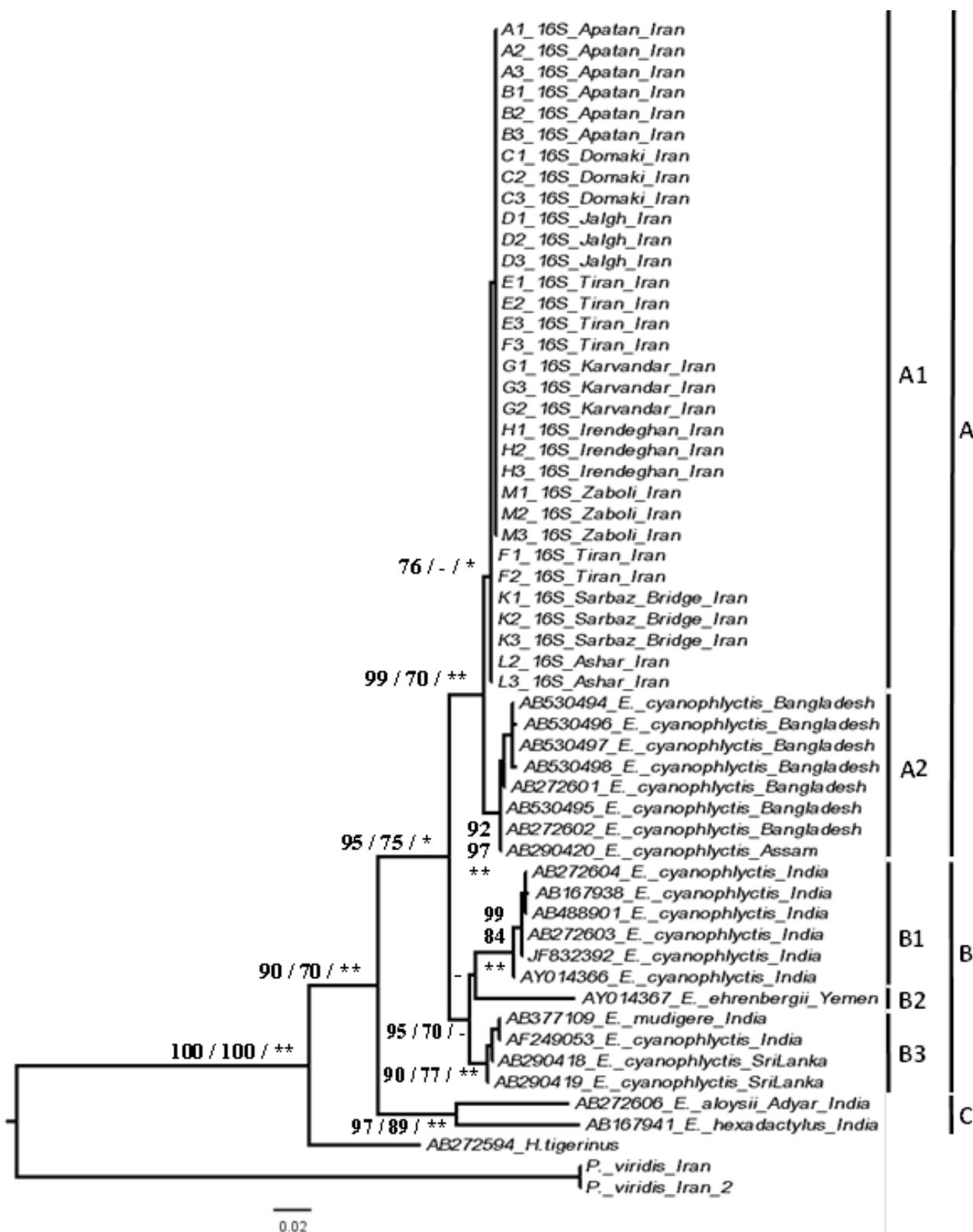
substitution rates (rate[A-C] 8.0429, rate[A-G] 15.1827, rate[A-T] 6.5556, rate[C-G] 0.9254, rate[C-T] 33.0808, rate [G-T]1.0000) estimated from the data set. These parameters were used for ML analysis.

The tree topologies are well resolved and identical among different analyses (Fig. 2). Two strongly supported primary clades (A/B and C) are recognized in all analyses. Clade (A) includes *E. cyanophlyctis* from Iran (A1) and *E. cyanophlyctis* from Bangladesh-Assam (A2). Clade (B) consists of *E. cyanophlyctis* from the southern India (B1), *E. ehrenbergii* (B2) beside *E. mudigere* and specimens from India and Sri Lanka that were previously identified as *E. cyanophlyctis* (B3) (Bossuyt and Milinkovitch, 2000; Alam et al., 2008). The third clade (C) comprises sequences of *E. aloysii* and *E. hexadactylus* (Fig. 2).

The sister relationship between the clade (A1) and (A2) was well supported across all optimality criteria (99% parsimony bootstrap value, 70% likelihood boot-

strap and 100% Bayesian posterior probability). In addition, our analyses reveal low genetic distances within each clade, including low inter-population genetic divergence between Iran and Bangladesh-Assam specimens (1.7%) and high divergence between Bangladesh-Assam and India (4.7%) (Table 2). Specimens of *E. cyanophlyctis* from Iran and the southern India had the lowest intra-population genetic distance (0.1%) while specimens from Bangladesh-Assam showed the highest intra-population genetic distance (0.5%) (Table 2).

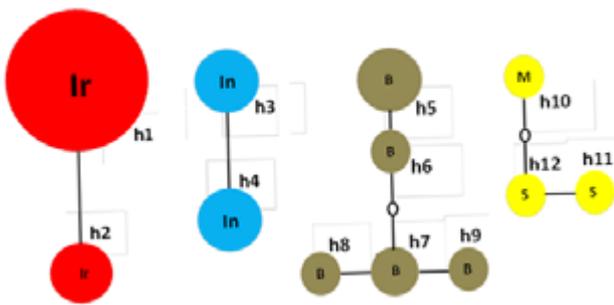
In total, 12 unique 16S haplotypes were identified in 49 individual skittering frogs. Low haplotype diversity was found in Iran populations (Hd= 0.353) in comparison to higher diversity found in the Bangladesh-Assam populations (Hd= 0.857). Additionally, the populations of skittering frogs from Iran demonstrate the lowest nucleotide diversity ( $\pi = 0.00072$ ) as compared to the populations from all the other regions. Bangladesh-Assam



**Fig. 2.** Maximum likelihood phylogeny for *Euphlyctis cyanophlyctis* (using a 492 bp portion of the mitochondrial 16S ribosomal RNA gene). Numbers represent MP and ML bootstrap values (MP/ML), respectively (500/5000 replicates). The posterior probability values from the Bayesian analysis are indicated at the > 99 % (\*\*) and > 95 % (\*) significance levels.

**Table 2.** P-distances of the 16S gene of *Euphlyctis* species. Within-group divergences in bold.

Species	1	2	3	4	5	6	7
1. <i>E. cyanophlyctis</i> (Iran)	<b>0.1</b>						
2. <i>E. cyanophlyctis</i> (Bangladesh-Assam)	1.7	<b>0.5</b>					
3. <i>E. cyanophlyctis</i> (India)	4.1	4.7	<b>0.1</b>				
4. <i>E. mudigere</i>	3.8	4.5	3.4	<b>0.4</b>			
5. <i>E. ehrenbergii</i>	6.7	7.4	5.4	5.1	-		
6. <i>E. hexadactylus</i>	9.9	10.3	9.8	9.9	11.5	-	
7. <i>E. aloysii</i>	10.8	10.7	11.1	10.2	11.5	7.7	-



**Fig. 3.** Haplotype networks of *Euphlyctis cyanophlyctis* (49 individuals) based on a 492 bp portion of the mitochondrial 16S ribosomal RNA gene. Networks were not joined if haplotypes were separated by more than 9 mutations. (h1, h2): Iran; (h3, h4): India; (h5, h6, h7, h8, h9): Bangladesh- Assam; (h10, h11, h12): Mudigere (AB377109), India and Sri Lanka.

populations have the highest nucleotide diversity ( $\pi = 0.00484$ ). Iran populations exhibit the lowest number of 16S rRNA haplotypes ( $H=2$ ) whereas the highest number of 16S rRNA haplotypes was found in Bangladesh-Assam ( $H=5$ ). These 12 haplotypes were grouped in four separate haplogroups that correspond to the geographic distribution of these samples (Table 1 and Fig. 3). The first haplogroup includes two haplotypes from Iran and the second haplogroup was identified as related to nominal *E. cyanophlyctis* from the southern India. The third haplogroup consists of five 16S rRNA haplotypes from Bangladesh-Assam specimens and the fourth haplogroup has three haplotypes that included specimens from Sri Lanka, southern India, and one specimen of *E. mudigere* (Fig. 3).

## DISCUSSION

The phylogenetic analyses, genetic distances, and haplotype network analyses strongly suggest that *E. cyanophlyctis* populations are split into four main genetic lin-

eages separated by a high level of nucleotide divergence (between 1.7% and 4.7% K2P distance). These lineages correspond to the eastern clade (A1) from Iran, the northern clade (A2) specimens Bangladesh-Assam, the southern clade (B1) that we consider to be nominal *E. cyanophlyctis* because of its proximity to the type locality, and the clade from southern India and Sri Lanka that includes *E. mudigere*.

Morphometric, morphological and molecular similarities (low haplotype and low nucleotide diversity) between populations of skittering frogs from southeastern Iran casts strong doubts on the validity of *E. cyanophlyctis seistanica*. In contrast, there is substantial divergence between populations of *E. cyanophlyctis* from Iran and the Indian subcontinent. Formation and subsequent sedimentation of the Sistan depression during the Neogene (Whitney, 2006) might have played a role as the main barrier, causing geographic isolation and divergence between populations of skittering frogs on the Indian subcontinent and in the Iranian Helmand Basin. Genetic divergence is a major consequence of vicariance and both dispersal events and isolation by distance can drive speciation (Lomolino et al., 2005). The formation of the Helmand barrier was proposed to have driven vicariance of different clades within the genus *Laudakia* and the *Eremias persica* complex (Macey et al., 1998; Rastegar Pouyani et al., 2010).

The close relationship of specimens from Iran and Bangladesh is difficult to explain in terms of geological and dispersal events. This could be the result of molecular homoplasy or ancestral state polymorphism (Avise, 1994) caused by recent fragmentation rather than recent dispersion. On the other hand, the specimens from Sri Lanka and India constitute a monophyletic group with *E. mudigere* suggesting that all of these specimens are referable to that species. The occurrence of *E. mudigere* in Sri Lanka and India was not unexpected since Sri Lanka is a part of the Deccan Plateau and was connected to the Indian subcontinent until the late Miocene (Deraniyagala, 1992). Although specific values of genetic divergence should not serve as the only basis for delimitation of species, a threshold of 3% genetic divergence could be used for recognition of a new species (based on Fouquet et al. 2007 for 16S). We recovered genetic divergences more than 3% between Bangladesh-Assam and southern Indian (4.7%) as well as between Iran and Indian populations (4.1%). These results are concordant with the study by Alam et al. (2008) that identified several cryptic species including at least four cryptic species currently identified as *E. cyanophlyctis*. These cryptic lineages are probably the result of allopatric speciation and a detailed taxonomic revision is strongly recommended.

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## REFERENCES

- Alam, M.S., Igawa, T., Khan, M.M.R., Islam, M.M., Kuramoto, M., Matsui, M., Kurabayashi, A., Sumida, M. (2008): Genetic divergence and evolutionary relationships in six species of genera *Hoplobatrachus* and *Euphlyctis* (Amphibia: Anura) from Bangladesh and other Asian countries revealed by mitochondrial gene sequences. *Mol. Phylogenet. Evol.* **48**: 515-527.
- Anderson, S.C. (1963): Amphibians and Reptiles from Iran. *Proc. Calif. Acad. Sci. Series* **431**: 417-498.
- Avise, J. C. (1994): *Molecular Markers, Natural History and Evolution*. Chapman and Hall, New York.
- Baluch, M., Kami, H.G. (1995): *Amphibians of Iran*. 2nd Ed. Tehran University Press, Tehran, Iran.
- Bauer, A.M. (1998): South Asian herpetological specimens of historical note in the Zoological Museum. *Berlin. Hamadryad* **23**: 133-149.
- Borkin, L.J., Litvinchuk S.N. (2013): Amphibians of the Palearctic: taxonomic composition. *Proc. Zool. Inst. AN* **317**: 494-541.
- Bossuyt, F., Milinkovitch, M. C. (2000): Convergent adaptive radiations in Madagascan and Asian ranid frogs reveal covariation between larval and adult traits. *Proc. Natl. Acad. Sci. USA* **97**: 6585-6590.
- Boulenger, G.A. (1920): A monograph of the South Asian, Papuan, Melanesian and Australian frogs of the genus *Rana*. *Rec. Ind. Mus.* **20**: 1-226.
- Clement, M., Posada, D., Crandall, K.A. (2000): TCS, a computer program to estimate gene genealogies. *Mol. Ecol.* **9**: 1657-1659.
- Deraniyagala, S.U. (1992): The prehistory of Sri Lanka: an ecological perspective. *Memoir 8*. 2nd ed. Archaeological Department, Colombo.
- Dubois, A. (1975): Un nouveau sous-genre (*Paa*) et trois nouvelles espèces du genre *Rana*. *Remarques sur la phylogénie des Ranidés (Amphibiens, Anoures)*. *Bull. Mus. Natl. Hist. Nat., Zool.* **3**: 1093-1115.
- Felsenstein, J. (1985): Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783-791.
- Fouquet, A., Gilles, A., Vences, M., Marty, C., Blance, M., Gemmel, N.J. (2007): Underestimation of species richness in neotropical frogs revealed by mtDNA analysis. *PLoS One* **2**: e1109.
- Frost, D.R. (2013): *Amphibian Species of the World: an Online Reference*. Version 5.6 (9 January 2013). Electronic Database accessible at [http:// research.amnh.org/herpetology/amphibia/index.html](http://research.amnh.org/herpetology/amphibia/index.html). American Museum of Natural History, New York.
- Hall, T.A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser.* **41**: 95-98.
- Hasan, M., Islam, M.M., Khan, M.M.R., Alam, M.S., Kurabayashi, A., Igawa, T., Kuaramoto, M., Sumida, M. (2012): Cryptic anuran biodiversity in Bangladesh revealed by mitochondrial 16S rRNA gene sequences. *Zool. Sci.* **29**: 162-172.
- Huelsenbeck, J.P., Ronquist, F.R., Teslenko, M. (2011): MRBAYES: Bayesian inference of phylogeny. <http://mrbayes.sourceforge.net/authors.php>.
- Josh, S.H., Alam, M.S., Kurabayashi, A., Sumida, M., Kuramoto, M. (2009): Two new species of the genus *Euphlyctis* (Anura, Ranidae) from southwestern India, revealed by molecular and morphological comparisons. *Alytes* **26**: 97-116.
- Khan, M.S. (1997): A new subspecies of common skittering frog *Euphlyctis cyanophlyctis* (Schneider, 1977) from Balochistan. *Pak. J. Zool.* **29**: 107-112.
- Khan, M.S. (2004): Annotated Checklist of Amphibians and Reptiles of Pakistan. *Asiat. Herpetol. Res.* **10**: 191-20.
- Kimura, M. (1980): A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Mol. Evol.* **15**: 111-120.
- Klootwijk, C.T., Pierce, J.W. (1979): India's and Australia's pole path since the late Mesozoic and the India Asia collision: *Nature* **282**: 605-607.
- Kosuch, J., Vences, M., Dubois, A., Ohler, A., Bohme, W. (2001): Out of Asia: mitochondrial DNA evidence for an Oriental origin of tiger frogs, genus *Hoplobatrachus*. *Mol. Phylogenet. Evol.* **21**: 398-407.
- Kotaki, M., Kurabayashi, A., Matsui, M., Kuramoto, M., Djong, T.H., Sumida, M. (2010): Molecular phylogeny of the diversified frogs of genus *Fejervarya* (Anura: Dicroglossidae). *Zool. Sci.* **27**: 386-395.

- Kurabayashi, A., Kuramoto, M., Joshy, H., Sumida, M. (2005): Molecular phylogeny of the ranid frogs from Southwest India based on the mitochondrial ribosomal RNA gene sequences. *Zool Sci.* **22**: 525-534.
- Librado, P., Rozas, J. (2009): DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451-1452.
- Lomolino, M.V., Riddle, B.R., Brown, J.H. (2005): *Biogeography*, 3rd edn. Sinauer Associates, Sunderland, MA.
- Macey, J.R., Schulte, J.A., Ananjeva, N.B., Larson, A., Rastegar-Pouyani, N., Shammakov, S.M., Papenfuss, T.J. (1998): Phylogenetic relationships among agamid lizards of the *Laudakia caucasia* species group: Testing hypotheses of biogeographic fragmentation and an area cladogram for the Iranian Plateau. *Mol. Phylogenet. Evol.* **10**: 118-131.
- Misonne, X. 1959. Zoogeographical analysis of mammals of Iran. *Mem. Roy. Inst. Nat. Sci. Belgium, Sec. Ser.* **59**: 1-157.
- Molnar, P., Tapponnier, P. (1975): Cenozoic tectonics of Asia—Effects of a continental collision. *Science* **189**: 419-426.
- Nikolskii, A.M. (1899): Reptiles, fishes, and Amphibia of the second expedition of N. A. Zarudni to Persia in 1898. *Ann. Mus. Zool. l'Acad. Impér. Sci. St. Pétersbourg*, **4**: 172-178, 375-417. [In Russian].
- Palumbi, S., Martin, A., Romano, S., McMillan, W. O., Stice, L., Grabowski, G. (1991): *The Simple Fool's Guide to PCR*. Version 2. Honolulu.
- Posada, D., Crandall, K.A. (1998): Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817-818.
- Rastegar Pouyani, E., Rastegar Pouyani, N., Kazemi Noureini, S., Joger, U., Wink. M. (2010): Molecular phylogeny of the *Eremias persica* complex of the Iranian plateau (Reptilia: Lacertidae), based on mtDNA sequences. *Zool. J. Linn. Soc.* **158**: 641-660.
- Rastegar-Pouyani, N., Kami, H.G., Rajabzadeh, M., Shafiei, S., Anderson, S.C. (2008): Annotated Checklist of Amphibians and Reptiles of Iran. *IJAB* **4**: 7-30.
- Sambrook, J., Maccallum, P., Russel, D. (2001): *Molecular cloning: A laboratory manual*, 3rd ed. Cold Springs Harbour Press, Cold Springs Harbour.
- Swofford, D.L. (2000): PAUP\*. Phylogenetic analysis using parsimony (\*and other methods). Version 4.0b4a. Sinauer, Sunderland, Mass.
- Tamura, K., Dudley, J., Nei, M., Kumar, S. (2007): MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**: 1596-1599.
- Thompson, J.D., Higgins, D.G., Gibson, T.J. (1994): Clustal-W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* **22**: 4673-4680.
- Whitney, J.W. (2006): *Geology, water, and wind in the lower Helmand Basin, Southern Afghanistan*. US Geological Survey Scientific Investigation Report.