First molecular identification of *Euphlyctis ehrenbergii* (Anura: Amphibia) inhabiting southwestern Saudi Arabia

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Abstract

The dicroglossid *Euphlyctis ehrenbergii* inhabiting southwestern Saudi Arabia has been identified morphologically and molecularly in this study. The morphometric measurements and their indices indicate that females are slightly bigger than males in some characters. Approximately 550 nucleotides of 16S rDNA gene were compared to the Yemeni haplotype available in the GenBank database. The phylogenetic inference revealed a strong sister relationship between the two haplotypes [bootstrap = 100% for maximum parsimony (MP), neighbor joining (NJ) and maximum likelihood (ML)]. The intra-specific genetic distance (\(D = 0.9\%\)) was lower than the observed variation between southern and northern haplotypes of *E. cyanophlyctis* (\(D = 4.6\%\)) currently ascribed to distinct lineages. Nonetheless, results suggest great intra-specific variation within *E. ehrenbergii*, deserving further investigation. The genetic distance between Yemeni and Saudi haplotypes of *E. ehrenbergii* (\(D = 0.9\%\)) resembles intra-specific distances between haplotypes of *E. cyanophlyctis* ascribed to the same cryptic lineages (e.g. as observed within the lineages including haplotypes from southern India and Sri Lanka). More samples and molecular data from both haplotypes are needed to better clarify their phylogenetic relationships. This study adds a clue for forensic herpetology by highlighting the efficiency of 16S rDNA gene in animal barcoding.

Keywords: Arabia, herpetology, Dicroglossidae, mtDNA, phylogeny

Introduction

The Dicroglossidae is an anuran family distributed in northwestern and sub-Saharan Africa, the southern Arabian Peninsula, and Asia throughout Pakistan, India and Bangladesh. The family contains 13 genera and 204 species (https://amphibiaweb.org, accessed 2018). Within the family, the genus *Euphlyctis* Fitzinger, 1843 inhabits southern and southwestern Asia. The fork-tongued frog *E. ehrenbergii* is distributed along the eastern coast of the Red Sea in the west of Saudi Arabia and Yemen throughout the Sarawat and Aseer mountains (Papenfuss et al. 2004). The species inhabits temporary and permanent freshwater bodies scattered at an altitudinal range up to 1400 m above sea level (asl) (Fitzinger 1843). Al-Qahtani (2011) recorded the species outside this range, in the east of the Sarawat Mountains. Southwestern Arabia is a mountainous region with severe climatic fluctuations (rain and temperature) and habitat fragmentation due to city expansion and new road construction, particularly during recent decades (Al-Obaid et al. 2017). Other factors such as wetland rarity and absence of rivers, lakes and marshes make the water body habitats temporary and subject the amphibian fauna to severe conditions (Al-Qahtani & Al-Johany 2018). The topographic features of the region are characterized by highland elevations. To the west, the mountains show a steep escarpment toward the Tihamah plain on the Red Sea coast. To the east, the mountains slope more gently to the inner sandy desert.
of the Empty Quarter. The escarpment runs in a north–south direction, parallel to and overlooking the Red Sea (Miller 1994). *Euphlyctis ehrenbergii* is widely distributed in the region, approximately 500 km north to Yemen and in North and South Yemen, being found in every fresh water body (Farag & Banaja 1980). As the Arabian Peninsula forms a bridge between the African and Eurasian continents, the Asir Mountains and the western highlands of Yemen provide an important bridge for floral and faunal biodiversity. This bridge allows the continuity of most vertebrate fauna to inhabit both Yemen and Southwestern Saudi Arabia.

Few morphometric and molecular systematic studies of *Euphlyctis* have been conducted so far. Based on the 16S rDNA gene, Khajeh et al. (2014) revealed new insights into the taxonomy of *E. cyanophlyctis*, partitioning it into four haplogroups of allopatric cryptic species. The same authors also revealed that *E. cyanophlyctis* is a species complex within which *E. ehrenbergii* is nested. Later, Priti et al. (2016) described a new species, *Euphlyctis karaavali*, from the southwest coast of India using molecular (12S and 16S rDNA genes) and morphological data. Other molecular investigations used the 16S rDNA gene fragment along with other mtDNA genes in addressing phylogenetic questions for the family Dicroglossidae (Kurabayashi et al. 2005; Alam et al. 2008; Howlader et al. 2015; Chen et al. 2017). The present study analyzed the same previously sequenced fragment of the 16S rDNA gene and conducted some morphometric analyses to identify Saudi *E. ehrenbergii* and compare it with the Yemeni haplotype.

### Materials and methods

#### Animals

Twelve adult males and females of *E. ehrenbergii* (six individuals of each sex) were collected from Wadi Alnadher (19°28′N, 41°51′E) of the Tihama plain in Southwestern Saudi Arabia (Figure 1) at an altitude of 710 m asl. Animals were collected according to the guidelines of the National Committee of Bio Ethics (NCBE). Samples were captured by hand from dam reservoirs and irrigated farms during the breeding season (spring) of 2017. All samples were deposited in the Zoology Department, Faculty of Science, King Saud University, Riyadh, Saudi Arabia.

#### Morphological study

Using a digital caliper, the morphometric parameters were measured to the nearest millimeter. Based on Priti et al. (2016), 14 morphometric parameters were measured: snout–vent length (SVL), foot length (FL), hind limb length (HLL), tympanum eye distance (TED), eye diameter (ED), tympanum diameter (TD), forelimb length (FAL), head length (HL), head width (HW), interorbital distance (IOD), internarial distance (IND), nostril–snout length (NSD), upper eyelid width (UELW) and first toe length (1st TL). From these parameters, nine indices were also measured as shown in Table I. These indices are NSL:SVL, ED:SVL, FL:SVL, HLL:SVL, FAL:SVL, IOD:SVL, HL:SVL, HW:SVL and IND:IOD. The morphometric measurements were compared using Student’s *t*-test for estimating the
Table 1. Morphometric measurements in mm (mean ± standard error) for male and female *Euphlyctis ehrenbergii*. Sample size is given in parentheses.

| Parameter | Male (6)           | Female (6)          | Significance |
|-----------|--------------------|---------------------|--------------|
| SVL       | 60.7 ± 5.30        | 71.9 ± 8.10         | p > 0.05     |
| FL        | 29.0 ± 1.80        | 33.9 ± 2.60         | p < 0.05     |
| HLL       | 50.4 ± 2.50        | 61.5 ± 7.60         | p < 0.05     |
| TED       | 1.9 ± 0.20         | 2.6 ± 0.35          | p < 0.01     |
| ED        | 7.2 ± 0.48         | 7.9 ± 0.84          | p < 0.05     |
| TD        | 4.2 ± 0.23         | 4.8 ± 0.32          | p < 0.05     |
| FAL       | 17.3 ± 1.60        | 22.1 ± 4.40         | p < 0.05     |
| HL        | 18.6 ± 1.30        | 22.7 ± 2.70         | p < 0.05     |
| HW        | 21.8 ± 1.50        | 27.9 ± 2.90         | p < 0.01     |
| IOD       | 2.7 ± 0.70         | 3.8 ± 0.65          | p < 0.05     |
| IND       | 2.2 ± 0.20         | 2.95 ± 0.30         | p < 0.01     |
| NSL       | 4.2 ± 0.48         | 5.2 ± 0.63          | p < 0.05     |
| UELW      | 4.1 ± 0.10         | 4.6 ± 0.50          | p > 0.05     |
| 1st TL    | 11.2 ± 0.80        | 13.3 ± 1.70         | p > 0.05     |
| NSL:SVL   | 0.07 ± 0.008       | 0.07 ± 0.003        | p > 0.05     |
| ED:SVL    | 0.12 ± 0.01        | 0.11 ± 0.004        | p < 0.05     |
| FL:SVL    | 0.49 ± 0.02        | 0.48 ± 0.03         | p > 0.05     |
| HLL:SVL   | 0.85 ± 0.06        | 0.86 ± 0.06         | p < 0.05     |
| FAL:SVL   | 0.29 ± 0.04        | 0.31 ± 0.05         | p > 0.05     |
| IOD:SVL   | 0.05 ± 0.01        | 0.05 ± 0.004        | p > 0.05     |
| HLL:SVL   | 0.30 ± 0.03        | 0.32 ± 0.03         | p < 0.05     |
| HW:SVL    | 0.36 ± 0.001       | 0.43 ± 0.06         | p > 0.05     |
| IND:IOD   | 0.88 ± 0.12        | 0.79 ± 0.08         | p > 0.05     |

Significant differences in each character between males and females. Difference becomes significant at p < 0.05.

**Molecular study**

Skeletal muscles were taken from the frog’s thigh, preserved in absolute ethanol and taken to the lab for further use. Only three individuals were genotyped using 16S rDNA. Approximately 100 mg of muscle tissue was cut into small pieces for DNA extraction using a Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The 16S rDNA gene was partially amplified using the forward primer 16SL: 5’-CGCCTGTATTATCAAAACAT-3’ and the reverse primer 16SH: 5’-CCGGTCTGAAAC TCAGATCACG-3’ (Palumbi et al. 1991). Polymerase chain reaction (PCR) was conducted as described by Amer et al. (2013). The amplified products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and the purified products were sequenced by Macrogen, Seoul, South Korea, according to their established protocol (http://www.macrogen.com/en/main/index.php). The sequenced fragment was deposited in the National Center for Biotechnology Information (NCBI) GenBank with accession numbers MH492263–MH492265.

The obtained sequence was dealt with using the BLASTN online program distributed on the NCBI homepage (http://www.ncbi.nlm.nih.gov) in order to find the most similar GenBank *Euphlyctis* sequences. The sequences were aligned using MacClade v. 4.10 (Maddison & Maddison 2002) in order to execute an aligned data file necessary for phylogenetic analysis. The pairwise alignments were conducted according to the method of Needleman and Wunsch (1970), and modified to deal with the more flexible costs allowed by MacClade. Ambiguous and gap-containing sites were excluded (Amer & Kumazawa 2005) from the aligned 550 bp, and the remaining sites (451 bp) were used for phylogenetic analyses under maximum-parsimony (MP), neighbor-joining (NJ) and maximum-likelihood (ML) methods in Paup v. 4.0b10 (Swofford 2002). For MP, the heuristic searches were conducted by tree bisection reconnection (TBR) branch swapping and 10 random additions with 10,000 bootstrap replications. The distance tree was done by the NJ method (Saitou & Nei 1987) using the Tamura–Nei distance option (Tamura & Nei 1993) and 10,000 bootstrap replications. The ML analysis was conducted after selecting...
the best fit with Modeltest v. 3 (Posada & Crandall 1998) using the Akaike information criterion (GTR +I + G). The heuristic search for ML was 10 random additions and nearest-neighbor interchange (NNI) branch-swapping with 500 bootstrap replications. Bootstrap replication was conducted by a single random addition sequence in all analyses. The taxa selected for phylogenetic analyses and their accession numbers are listed in the inferred tree (Figure 2). Two samples of *Bufotes viridis* (KF992832, KF992833) were used as outgroups.

Pairwise distance analysis was conducted using the Tamura–Nei model (Tamura & Nei 1993). This analysis involved 22 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

### Results and discussion

The morphometric characters and their indices indicated that the body length of the adult frog ranges from SVL = 55 to 80 mm (Table I). Some characters exhibited significant differences between sexes, being larger in females. These characters were FL, HLL, TD, HL, IOD (p < 0.05), TED, HW and IND (p < 0.01). For the indices, only ED:SVL exhibited a significant difference between females and males (p < 0.05).

At the molecular level, three samples showed identical sequences for the targeted fragment (i.e. one single haplotype). In the final alignment, four base substitutions between Yemeni and Saudi haplotypes were found. Ambiguous (noisy) and gap-containing sites were removed, such that 451 bp were kept for the phylogenetic analysis. Overall, 303 nucleotide positions were constant, 126 sites were informative under parsimony criteria and 22 were parsimony uninformative. The parsimony analysis showed consistency index (CI) = 0.734, homology index (HI) = 0.266, CI excluding uninformative sites = 0.709, HI excluding uninformative sites = 0.291, retention index (RI) = 0.823 and rescaled consistency index (RC) = 0.604. In the phylogeny, the newly isolated haplotype from Saudi Arabia acquired a strong sister relationship with the Yemeni haplotype of *E. ehrenbergii* (bootstrap = 100% for MP, NJ and ML; Figure 2). This sister relationship was strongly supported by MP (bootstrap = 97%) and NJ (bootstrap = 100%) and reasonably supported by ML (bootstrap = 75%).

Table II shows the pairwise genetic distance matrices (%) among closely related congeneric *E. ehrenbergii* taxa. Distances between haplotypes ascribed to the same lineages are the lowest. *Euphlyctis ehrenbergii* from southwestern Saudi Arabia is differentiated from Yemeni *E. ehrenbergii* by 0.9%. *Euphlyctis cyanophlyctis* from the northern clade (North India, Bangladesh and Iran) exhibited a distance with lower limit of 4.6% from *E. cyanophlyctis* of the southern clade (South India and Sri Lanka).

Using the same gene fragment, a recent study (Khajeh et al. 2014) split southwestern Asian *Euphlyctis* into four lineages of Iran, Bangladesh and India, and a lineage of southern India and Sri Lanka. In the present study, as was found by Khajeh et al. (2014), Iranian and Bengali lineages formed one northern clade while Indian and Sri Lankan lineages formed a southern clade, and the two clades are sister to each other. This clustering is geographically meaningful, since each clade acquires homogeneity in climate and geography.
Table II. Genetic distance matrix (%) calculated using the Tamura–Nei model based on 16S sequences between *Euphlyctis* species and haplotypes. For details of species and haplotypes used, refer to Figure 2 showing the taxa and their accession numbers.

| Accession no. | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  | 19  | 20  | 21  |
|--------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1            | Saudi haplotype | 0.9 |
| 2            | Yemeni haplotype | 8.5 |
| 3            | EU367011         | 15.6 |
| 4            | KU179086         | 6.3 |
| 5            | AB290418         | 9.4 |
| 6            | AB272602         | 5.3 |
| 7            | KU179082         | 8.1 |
| 8            | KF992818         | 8.9 |
| 9            | KF992800         | 8.9 |
| 10           | KF992827         | 8.7 |
| 11           | KF992806         | 8.9 |
| 12           | AB272603         | 6.9 |
| 13           | AB272604         | 8.1 |
| 14           | AB272606         | 14.7 |
| 15           | AB272594         | 14.8 |
| 16           | AB377109         | 5.4 |
| 17           | AB176941         | 15.6 |
| 18           | AB272601         | 9.1 |
| 19           | AB530494         | 9.4 |
| 20           | KF992832         | 27.6 |
| 21           | KF992833         | 27.6 |

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Regarding *E. ehrenbergii*, the Yemeni haplotype was located, in a previous phylogeny, within the southern clade, indicating the paraphyly of *E. cyanophlyctis* (Khajeh et al. 2014). In the present study, Saudi and Yemeni *E. ehrenbergii* appears nested within the southern clade of *E. cyanophlyctis* (Figure 2). The current study also found a genetic distance between Yemeni and Saudi *E. ehrenbergii* lower than that found between haplotypes assigned to the southern and northern *E. cyanophlyctis* (Khajeh et al. 2014). This distance (< 1%) is very close to the minimum threshold identifying new candidate species (Li et al. 2016). It suggests great intra-specific variation, which possibly could underlie the occurrence of cryptic taxa along the distributional range of *E. ehrenbergii*. More samples/localities/markers (including nuclear DNA genes) should be investigated to shed light on the systematics of the species and its populations. A recent molecular and morphological study (Priti et al. 2016) on describing new *Euphlyctis* species showed a phylogenetic situation of *E. ehrenbergii* similar to that constructed in the present study.

Finally, it should be noted that there is a direct link between wildlife and forensics, particularly for endangered animals since they are sometimes considered as the subject of a legal case. Some of the herpetofauna are threatened and have been seen in trade. The phylogeographic characterizations of these convention on international trade in endangered species of wild fauna and flora (CITES)-protected animals could provide baseline data for forensic herpetology (Baker et al. 2012). The Arabian *E. ehrenbergii* is not of conservation concern to zoologists, and it is not recorded in international union for conservation of nature (IUCN 2018) as a trade animal. However, it exhibited genetic variability, perhaps because of the absence of permanent water bodies (Al-Qahtani & Al-Johany 2018), and it could, therefore, be threatened in the near future. It is thus important to study variation of 16S rDNA, as this gene could be used as an effective tool for forensic authentication (Naga Jogayya et al. 2013).

In conclusion, genetic variability between Saudi and Yemeni *E. ehrenbergii* was similar to that found between southern Sri Lanka and Indian *E. cyanophlyctis*. This finding indicates that both Saudi and Yemeni *E. ehrenbergii* need further molecular investigation to address their phylogenetic and systematic situations.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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