

# FIRST MOLECULAR MITOCHONDRIAL D-LOOP PHYLOGENY OF *SCLEROPHRYS TIHAMICA* (BALLETO AND CHERCHI, 1973) (ANURA: BUFONIDAE) IN SAUDI ARABIA

ALQAHTANI, A. R.

Department of Biology, College of Science, University of Bisha, Bisha 61922, Saudi Arabia  
(e-mail: arabe@ub.edu.sa)

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**Abstract.** The molecular phylogeny of *Sclerophrys tihamica* (Balletto and Cherchi, 1973) is presented to understand the evolutionary relationships among amphibian species in the region. Eleven *Sclerophrys tihamica* toad samples were collected from Tihama, Saudi Arabia. Our analysis supports the paraphyly of the genus *Sclerophrys*, with *S. tihamica* and *D. dhufarensis* as sister groups from Saudi Arabia and *S. arabica*, *Bufotes viridis* from Kazakhstan, and *Sclerophrys regularis* from Egypt. The genetic distances among the species were also calculated, and they had varied levels of divergence. Further analysis with a larger and more representative sample of species is needed to confirm these findings. The study highlights the importance of understanding the evolutionary relationships among amphibians in Saudi Arabia for their conservation and the sustainability of local ecosystems.

**Keywords:** phylogenetic analysis, Bufonidae, Saudi Arabia, Yemen, phylogeography

## Introduction

Amphibians are a diverse class of vertebrates that inhabit various ecological environments, although their role in ecosystem dynamics remains understudied (West, 2018; Al-Johany et al., 2014). However, their potential abundance suggests significant energy transfer and nutrient cycling roles (Gaston et al., 2018). In addition, their life history, diversity, and behaviors play a vital role in assessing ecological and evolutionary theories (Myers et al., 2000; Vitt and Caldwell, 2013). These amphibian species are important components of the biodiversity of Saudi Arabia and their conservation is crucial for the health of local ecosystems (Al-Johany et al., 2014; Al-Qahtani and Al-Johany, 2018).

Seven amphibian species have been recorded in Saudi Arabia, belonging to four families (Al-Qahtani and Al-Johany, 2018; Frost, 2023). The Bufonidae family includes four species: *Sclerophrys arabica* (Heyden, 1827), *S. tihamica* (Balletto and Cherchi, 1973), *Bufotes sitibundus* (Pallas, 1771), and *Duttaphrynus dhufarensis* (Parker, 1931). The Hylidae family is represented by only one species, *Hyla felixarabica* (Gvoždík et al., 2010). The Ranidae family is represented by *Pelophylax ridibundus* (Pallas, 1771). Lastly, the Dicroglossidae family is represented by *Euphlyctis ehrenbergii* (Peters, 1863). *S. arabica* is the most common toad species distributed in the Arabian Peninsula (Al-Johany et al., 2014; Al-Qahtani, 2011; and Aloufi et al., 2019). It inhabits places near water sources such as springs, oases, and irrigation channels (Balletto et al., 1985). Additionally, *S. tihamica* is an endemic species to Arabia and occurs along the coastal plain of the Red Sea of Saudi Arabia and Yemen. The above species is known previously as *Bufo pentoni*. The Dhofar toad *D. dhufarensis* or “Oman toad” occupies places with water bodies such as springs, pools, dams, and oases without any threats of extinction (Soorae et al., 2013). The African common toad *S. regularis* is widely distributed in Sub-Saharan Africa, and its range extends from Algeria and Libya, as well as northern Nilotic Egypt (Rödel, 2000; Poynton et al., 2016).

Only a limited number of molecular systematic studies have been conducted on *Sclerophrys* (Alrefaei, 2022) that focused on the 16S rDNA gene. Nevertheless, these studies are scarce and have not covered most regions of the country (Hafez et al., 2017; Alqahtani and Amer, 2019). The present study represents the first attempt to document the phylogenetic relationship of *Sclerophrys tihamica* from Saudi Arabia, utilizing comparative sequence data available in GenBank based on the D-loop gene. Aso, to enhance our understanding of the evolutionary connections within this genus and provide insights into its taxonomic classification.

## Material and methods

### Study area

The Tihamah plain is a coastal region extending approximately 800 kilometers from Jeddah in the north to the Yemen border in the south. This area is characterized by its low elevation and high temperatures, often reaching around 40°C. The southern parts of Tihamah are situated within a coastal fog desert zone, where temperatures can soar to 43°C, accompanied by relative humidity levels ranging from 40% to 60%. These unique environmental conditions contribute to a distinct habitat for the species *S. tihamica*. The high temperatures and specific humidity levels may significantly affect the species' behavior, physiology, and ecological interactions. Notably, the coastal fog desert zone plays a crucial role in supporting a specialized ecosystem that influences the distribution and survival of various species.

### Materials

Eleven *S. tihamica* toads were collected from Tihama, southwestern Saudi Arabia (Table 1; Fig. 1), according to ethical considerations regarding wildlife sampling, and by using the toe-clipping technique, as it is a standard technique in herpetological studies to ensure accurate identification and minimize harm to the animals (Halliday, 1996). Tissue samples were taken from toads caught by hand and fishing nets in streams and pools. After tissue sampling, the toads were preserved in 95% ethanol and stored at 4°C. Total DNA was extracted from tissue samples using a standard phenol/chloroform method and proteinase K (Kirby, 1993). A fragment of the mitochondrial gene D-loop was amplified by PCR using the primers and conditions of Goebel and Gruenwald (1999) and Hafez et al. (2017). The sequences were edited using Chromas v.1.43, checked for accuracy, and aligned individually with the opposite strand from the same individual using the program. All sequences were deposited in GenBank (Fig. 1; Table 1).

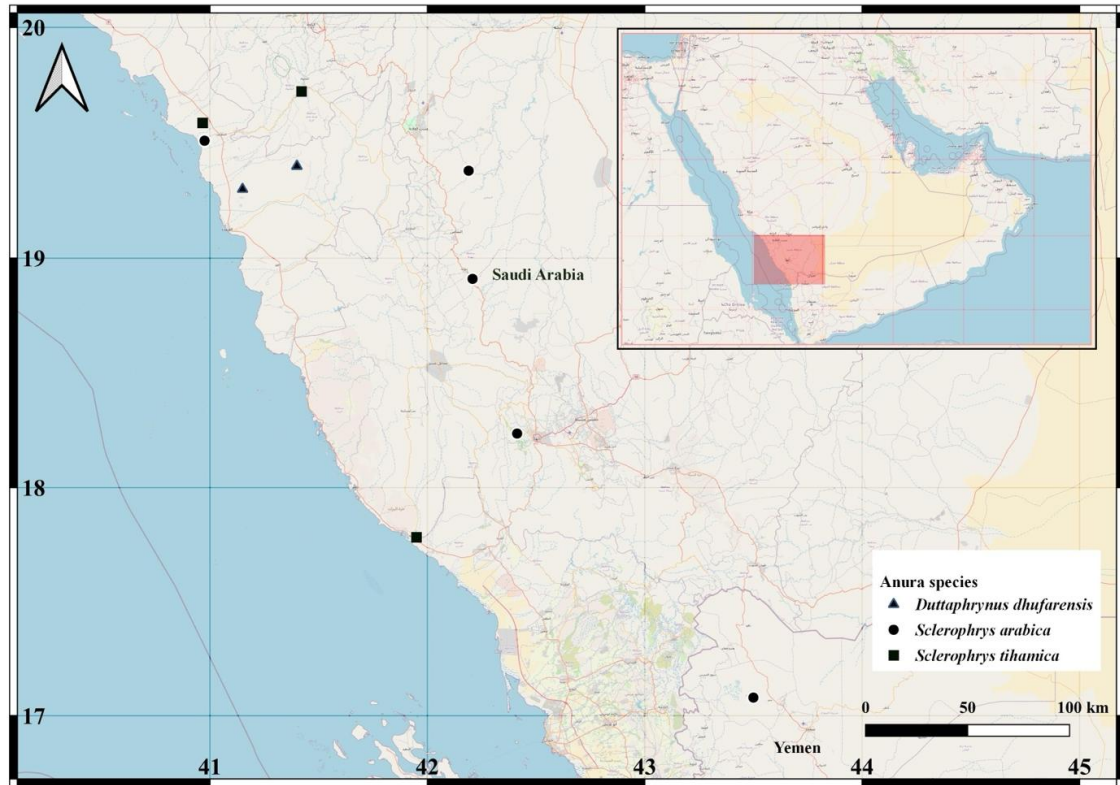
Phylogenetic analyses were performed using Finch TV (Finch TV 1.4.0, Geospiza, Inc., USA; www.geospiza.com). The Dloop sequences of *Bufo viridis*, *D. dhufarensis*, *S. regularis*, and *S. arabica* (Table 1) were retrieved and downloaded from the gene bank of the Middle East region as in groups, with *Fejervarya limnocharis* (KF051071.1), used as outgroup.

The data set was aligned using the default settings of ClustalW in Mega 11, and the nucleotide composition was calculated based only on the ingroup sequences. Pairwise genetic distances were computed to estimate the sequence divergence for the entire data set Tamura and Nei model of evolution (Tamura et al., 2021). Maximum-parsimony and neighbor-joining analyses for the aligned data set were performed with Paup version 4.0b3a (Swofford, 2000), with a heuristic search option using stepwise addition followed

by tree bisection reconnection (TBR) branch swapping. The gaps were treated as missing characters in all alignments, and confidence in the nodes was assessed through 1000 bootstrap replicates (Felsenstein, 2002). The geographical structure was inferred using Bayesian inference implemented with MrBayes 3.1.2. The best-fit models of nucleotide evolution or Bayesian inference analyses were utilized to select based on the Akaike information criterion using MrModeltest 2.3 (Nylander, 2004) (HKY, AIC) (Akaike, 1973). The analyses were run for two million generations, and the output parameters were evaluated to ascertain the stationarity and convergence of MCMC using Tracer 1.4 (Rambaut et al., 2018).

**Table 1.** Records of *Sclerophrys tihamica* and GenBank sequences used in phylogenetic studies, including their accession numbers submitted to GenBank

No	Species	Acc. No	Lat.	Long.	Site	Authority
1	<i>S. tihamica</i>	KU728664.1	19.586694	40.9664	Tihama, KSA	This study
2	<i>S. tihamica</i>	KU728663.1	19.586694	40.9664	Tihama, KSA	This study
3	<i>S. tihamica</i>	KU728662.1	19.586694	40.9664	Tihama, KSA	This study
4	<i>S. tihamica</i>	KU728661.1	19.586694	40.9664	Tihama, KSA	This study
5	<i>S. tihamica</i>	KU728660.1	19.723256	41.4213	Tihama, KSA	This study
6	<i>S. tihamica</i>	KU728659.1	19.723256	41.4213	Tihama, KSA	This study
7	<i>S. tihamica</i>	KU728658.1	19.723256	41.4213	Tihama, KSA	This study
8	<i>S. tihamica</i>	KU728657.1	19.723256	41.4213	Tihama, KSA	This study
9	<i>S. tihamica</i>	KU728656.1	17.783	41.9499	Tihama, KSA	This study
10	<i>S. tihamica</i>	KU728655.1	17.783	41.9499	Tihama, KSA	This study
11	<i>S. tihamica</i>	KU728654.1	17.783	41.9499	Tihama, KSA	This study
12	<i>S. arabica</i>	KJ094431.1	18.0099	43.4599	Abha, KSA	This study
13	<i>S. arabica</i>	KJ094430.1	18.0099	43.4599	Abha, KSA	This study
14	<i>S. arabica</i>	KJ094429.1	18.0099	43.4599	Abha, KSA	This study
15	<i>S. arabica</i>	KJ094428.1	18.0099	43.4599	Abha, KSA	This study
16	<i>S. arabica</i>	KJ094427.1	19.5101	40.9751	Tihama, KSA	This study
17	<i>S. arabica</i>	KJ094426.1	19.5101	40.9751	Tihama, KSA	This study
18	<i>S. arabica</i>	KJ094425.1	19.5101	40.9751	Tihama, KSA	This study
19	<i>S. arabica</i>	KJ094424.1	19.5101	40.9751	Tihama, KSA	This study
20	<i>S. arabica</i>	KJ094423.1	19.5101	40.9751	Tihama, KSA	This study
21	<i>S. arabica</i>	KJ094422.1	19.5101	40.9751	Tihama, KSA	This study
22	<i>S. arabica</i>	KJ094421.1	19.3800	42.1899	Wadi_Tarj, KSA	This study
23	<i>S. arabica</i>	KJ094420.1	19.3800	42.1899	Wadi_Tarj, KSA	This study
24	<i>S. arabica</i>	KJ094419.1	18.6600	41.2998	Tanomah, KSA	This study
25	<i>S. arabica</i>	KJ094418.1	18.6600	41.2998	Tanomah, KSA	This study
26	<i>S. arabica</i>	KJ094417.1	18.6600	41.2998	Tanomah, KSA	This study
27	<i>S. arabica</i>	KJ094416.1	18.6600	41.2998	Tanomah, KSA	This study
28	<i>S. arabica</i>	KJ094415.1	18.6600	41.2998	Tanomah, KSA	This study
29	<i>S. arabica</i>	DQ629592.1	17.0800	43.4997	Saraa	Stoeck et al., 2006
30	<i>D. dhufarensis</i>	KM289094.1	19.3999,	41.4000	West Almukhwah, KSA	Hafez et al., 2015
31	<i>D. dhufarensis</i>	KM289095.1	19.3999,	41.4000	West Almukhwah, KSA	Hafez et al., 2015
32	<i>D. dhufarensis</i>	KM289096.1	19.3999	41.3998	Wadi Alahsabab, KSA	Hafez et al., 2015
33	<i>D. dhufarensis</i>	KM289097.1	19.3000	41.14992	Wadi Alahsabab, KSA	Hafez et al., 2015
34	<i>B. viridis</i>	DQ629801.1	48.540014	69.279861	Kazakhstan	Stoeck et al., 2006
35	<i>S. regularis</i>	KU291414.1	30.0600	31.0799	Egypt	Hafez et al., 2015
36	<i>S. regularis</i>	KU291408.1	30.0600	31.0799	Egypt	Hafez et al., 2015



**Figure 1.** Map showing where *Sclerophrys tihamica* samples from Saudi Arabia and GenBank sequences used in phylogenetic studies were collected (see Table 1 for location details)

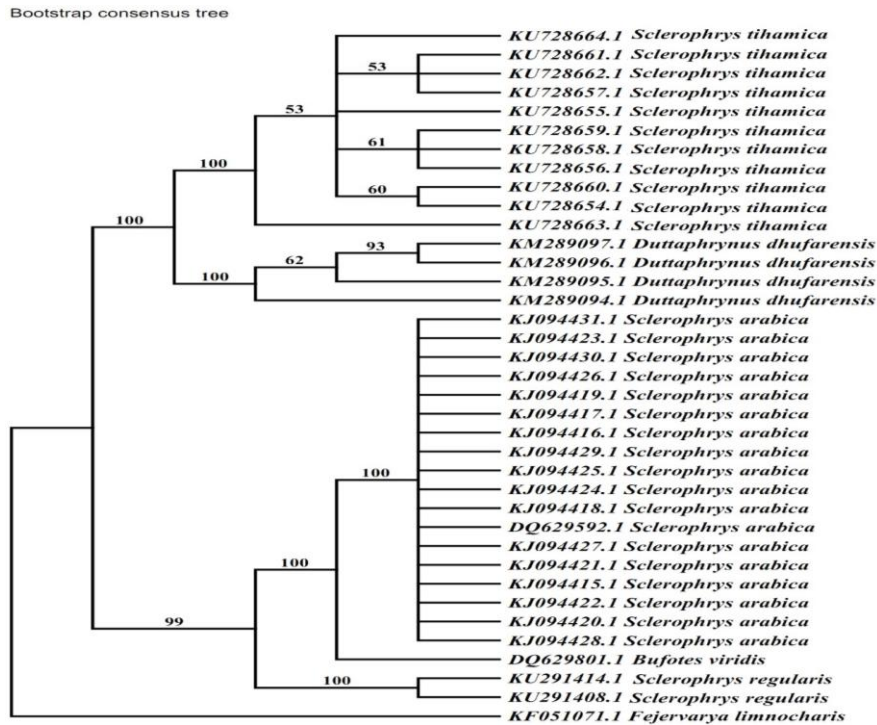
## Results

### Sequence data

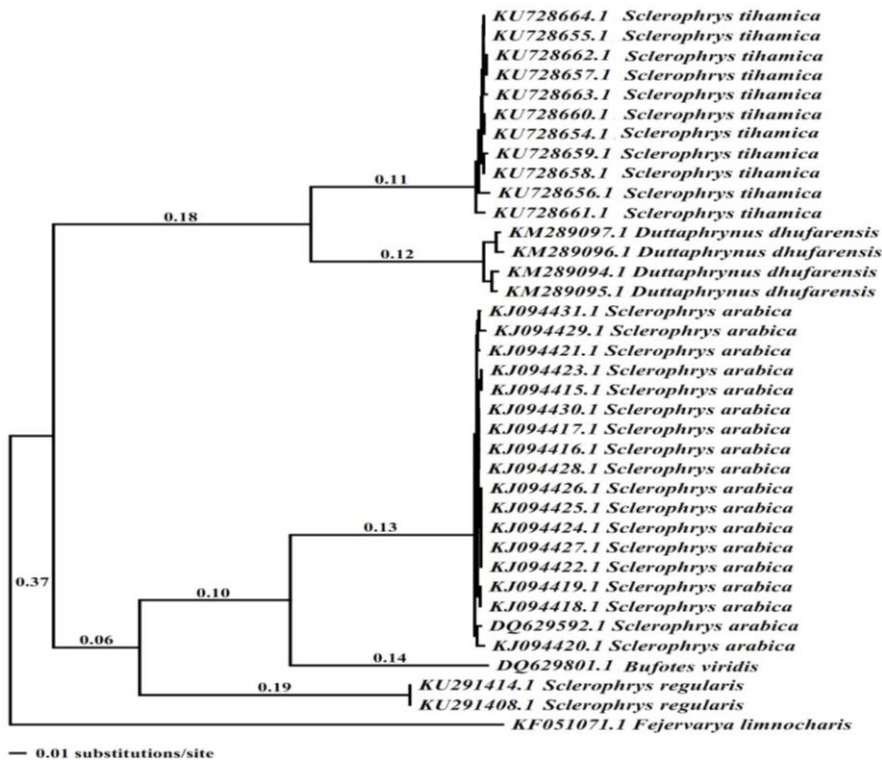
The dloop gene dataset contained 467 aligned nucleotides. Among these, 43 (9.2%) bases remained constant, while 421 (90%) were variable and 358 (76%) were informative for parsimony. Within the 368 bp, 84 polymorphic segregating sites were identified. Sequence divergences among ingroup lineages varied from 0.00 to 0.60, with an average of 0.1082. All sequences demonstrated typical mitochondrial nucleotide composition, with average percentages of A = 33.1%, C = 16.1%, G = 13.5%, and T = 37.3%.

### Phylogenetic tree construction

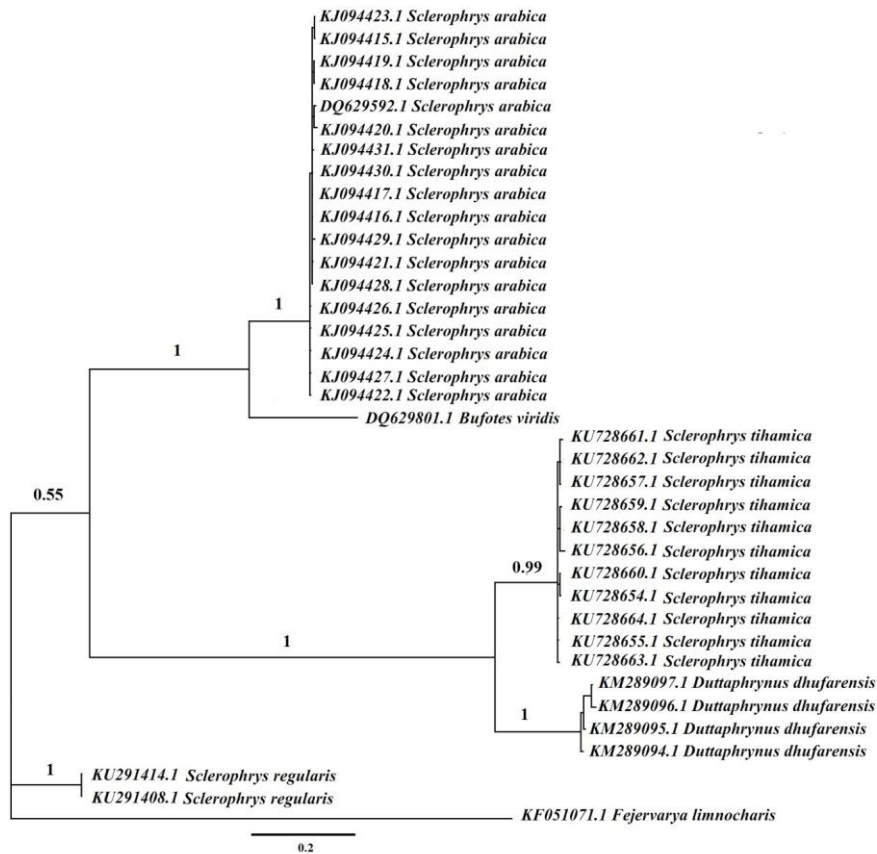
Maximum parsimony used 467 bp sequences, treating gaps as missing data, resulting in a 322-step tree. The tree's characteristics were homoplasy index = 0.142, consistency index = 0.858, and retention index = 0.972. The maximum-parsimony tree topology (Fig. 2) matched the neighbor-joining tree (Fig. 3) but slightly differed from Bayesian analyses (Fig. 4). In all phylogenetic analyses (Figs. 2–4), two well-supported clades included all *Sclerophrys*, *Buteous*, and *Duttaphrynus* taxa. The first clade grouped *S. tihamica* from Tihama with *Duttaphrynus dhufarensis* from Wadi Alahsabab and West Almukhwah, Asir, Saudi Arabia (Bootstrap = 100% and posterior probability = 1). The second clade grouped *S. arabicus* from Saudi Arabia and Yemen (Saraa) with *B. viridis* from Kazakhstan and *S. regularis* from Egypt.



**Figure 2.** Phylogenetic tree using maximum parsimony method, illustrating the relationship between *Sclerophrys tihamica* and GenBank sequences based on Dloop sequences. The numbers above the branches represent bootstrap values



**Figure 3.** Phylogenetic tree using Neighbor-Joining method, illustrating the relationship between *Sclerophrys tihamica* and GenBank sequences based on Dloop sequences. The numbers above the branches indicate distance values



**Figure 4.** Bayesian inference tree illustrating the relationship between *Sclerophrys tihamica* and GenBank sequences based on Dloop sequences. The numbers above the branches represent bootstrap values

### Genetic distance analysis

Table 2 shows the genetic distances from the entire data set. The distance between *S. tihamica* and *D. dhufarensis* had the lowest divergence (24%). However, the distance between *S. tihamica* and *S. arabica* was 59%. The highest divergence was observed between *S. tihamica* and *B. viridis* species, which was 60%.

### Discussion

Our phylogenetic analyses support the paraphyly of the genus *Sclerophrys* (Figs. 2–4) based on the Dloop gene, representing different species of *Buteous*, and *Duttaphrynus*. Graybeal (Graybeal, 1997) previously proposed the paraphyletic relationship of the genus *Bufo* (= *Sclerophrys*) through molecular analysis of DNA samples collected from various populations across the geographic and taxonomic range of the Bufonidae family. Portik and Papenfuss (2015) suggested no close relationship exists between these Arabian lineages (*Sclerophrys* and *Duttaphrynus*). However, our molecular data indicate the paraphyly of *Sclerophrys* species. Further analyses with more representative species should be conducted to confirm this finding.

Meanwhile, the current phylogeny shows that *S. tihamica* and *D. dhufarensis* are sister groups from Saudi Arabia, while *S. arabica*, *Bufo viridis* from Kazakhstan, and *S.*

*regularis* from Egypt. Also, our analysis revealed a genetic divergence between *S. tihamica* and samples of *D. dhufarensis* and *S. arabica* from Saudi Arabia (p-distance = 24%, 59%, respectively). Also, the phylogenetic trees were topologically consistent (Figs. 2–4), and we found that *S. tihamica* was most closely related to the *D. dhufarensis* sequence from Saudi Arabia and Yemen. Our results agree with Hafez et al. (2017) which revealed that *D. dhufarensis*, and *S. arabica* showed a close relationship to the Asian species *D. melanostictus* from China and *D. stomaticus* from Pakistan, according to the D-loop gene sequence and *D. melanostictus* from Indonesia according to the coding gene fragment 12S rRNA. Similarly, Alrefaei et al. (2022) confirmed the close relationship between *S. arabica* and *D. dhufarensis*.

**Table 2.** Pairwise distances among *Sclerophrys tihamica* and GenBank sequences used in phylogenetic studies based on Dloop sequences, with standard error estimates above the diagonal

Taxa / group	<i>S. tihamica</i>	<i>S. arabica</i>	<i>D. dhufarensis</i>	<i>B. viridis</i>	<i>S. regularis</i>	Out group
<i>S. tihamica</i>		0.023	0.021	0.023	0.023	0.023
<i>S. arabica</i>	0.590		0.024	0.021	0.023	0.022
<i>D. dhufarensis</i>	0.248	0.597		0.024	0.024	0.023
<i>B. viridis</i>	0.600	0.267	0.616		0.023	0.023
<i>S. regularis</i>	0.571	0.422	0.582	0.435		0.024
Out group	0.656	0.665	0.682	0.637	0.583	

Moreover, Frost et al. (2006) suggested that *S. tihamica* is not a member of the monophyletic taxon Bufo but could not allocate it to any other genera, so it was left in a non-taxon (Bufo). On the contrary, Portik and Papenfuss (2015) found that *S. arabica* and *S. tihamica* diverged from their closest African relatives in the early Miocene, around 20.5-21.2 Mya, and did not colonize the Arabian Peninsula. Geologic events related to the formation of the Red Sea may have altered the genetic pool of Afro-Arabian toad species and contributed to their divergence (Wong et al., 2010). The formation of the Red Sea stands out as a pivotal event that has shaped the evolutionary landscape for amphibians in this region. However, the understanding this historical biogeographical event not only enriches our comprehension of *Sclerophrys* phylogeny, but also underscores the importance of conservation efforts aimed at protecting these unique amphibian populations amidst ongoing environmental changes. The shared distributional patterns and migrations, along with the phylogenetic data, may shed light on the historical components that explain the evolution and colonization of the regional fauna (Cracraft and Prum, 1988; Cracraft, 1994). Fortunately, amphibians in Saudi Arabia are adaptable to various environments and modified habitats, so they do not face serious threats (Al-Johany et al., 2014; Al-Qahtani, 2011). Our hypothesis implies different biogeographic scenarios to explain the current geographical distribution of these species.

## Conclusion

The molecular data analyzed in this study supports the paraphyly of the genus *Sclerophrys*. The current phylogeny indicates that *S. tihamica* and *D. dhufarensis* are sister groups from Saudi Arabia, while *S. arabica*, *Bufotes viridis* from Kazakhstan, and

*Sclerophrys regularis* from Egypt. Further analysis with a more representative sample of species is needed to confirm these findings. The shared distributional patterns and the phylogenetic data will help explain the evolution and colonization of the regional fauna. This study highlights the genetic divergence among *Sclerophrys tihamica* and other related species, which indicates that regions with high genetic diversity are essential for maintaining healthy amphibian populations. Genetic management conservation strategies should prioritize these areas to ensure the survival of various genetic lineages, which can enhance resilience to environmental changes and disease.

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**Conflict of interests.** The author declares there is no conflict of interests.

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