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ORIGINAL ARTICLE

Phylogenetic relationships and divergence times of the poorly known genus *Spalerosophis* (Serpentes: Colubridae)

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Abstract
Spalerosophis Jan, 1865, is a colubrid snake genus distributed in arid and semiarid regions from northwestern Africa to northern India. Six species of *Spalerosophis* are identified based on traditional morphology; however, there are arguments about their systematic status. We performed the first molecular phylogenetic analyses for this genus using nucleotide sequences of c. 2278 bp from three mitochondrial genes (12S rRNA, 16S rRNA, and cyt b) to investigate the phylogenetic relationships and historical evolution of the poorly known *Spalerosophis* species and doubtful populations. We used fossil calibrations for dating divergences. Our phylogenetic analyses clearly showed five separate species with high support values, including *S. arenarius*, *S. atriceps*, *S. microlepis*, *S. diadema schirasianus*, and *S. diadema cliffordii*. Bayesian calibrated molecular clock suggested that the genus *Spalerosophis* diverged from the common ancestor in the Paleogene around 35.28 Mya (25.89–44.66). Our analysis indicated that isolated *S. atriceps* was separated around 21.72 Mya (10.86–33.04) as the basal diverged species of the genus. This suggested that the species of *Spalerosophis* probably originated from an ancestor somewhere in the Iranian Plateau and then dispersed to its current geographical range. *Spalerosophis arenarius* and *S. atriceps* were identified as the two last diverged members of the genus, separated from *S. d. schirasianus* during the Middle Miocene about 13.31 Mya (5.52–22.33). These results indicate that the separation of *Spalerosophis* species coincided with the orogenic events of the Zagros Mountains in western Iran and the Sulaiman Mountains in Afghanistan, India, and Pakistan. Their diversification may therefore be the result of vicariance events that promoted their current distribution. This work provides a foundation for future studies on the phylogeny, diversity, and evolution of the genus *Spalerosophis* and highlights the need for more molecular studies on unknown snakes.

Keywords *Spalerosophis* · Colubridae · Mt DNA · Phylogeny · Snake

Introduction

Classification and phylogenetic relationships of snakes have always been under debate. The limited range of morphological characters, the enormous diversity of living species (> 3920 species), and the limited taxonomic and genomic sampling in molecular phylogenetic studies have been the main deterrents to significant advances in understanding snake phylogeny (Uetz et al., 2021; Zaher et al., 2009). Although the classification and phylogeny of many taxa have had significant progress over the last decades, there are still some understudied critical taxa. Genus *Spalerosophis* is one of them. The genus *Spalerosophis* established by Jan (1865) belongs to a large and diverse radiation of colubrid snakes, the Colubrinae, which occurs in arid and semiarid regions from northwestern Africa to northern India; its Saharo-Sindian range area consists of three separate regions: Afro-Arabia, Irano-Turan, and Indo-Pakistan (Marx, 1959). *Spalerosophis* spp. possess four particular and common traits that include the following: (1) the orbit surrounded by oculars and separated upper labials from the orbit, (2) entire anal plate (except in *S. josephscorteccii*), (3) a high number of temporal scales, and (4) prefrontals and loreals broken up into small scales (Baig & Masroor, 2008; Lanza, 1964; Minton, 1966).

During the last 150 years, attempts have been made to evaluate the taxonomy of the genus *Spalerosophis* mostly based

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on morphological evidences, and the systematic concept of this genus has been under modification (Baig & Masroor, 2008; Marx, 1959; Schatti et al., 2010). *Spalerosophis* Jan, 1865, was established as a monotypic genus for *S. microlepis* from western Iran. Boulenger (1893) synonymized *Spalerosophis* Jan with *Zamenis* auct. Schmidt (1930) revalidated the genus for three species, including the *S. diadema*, *S. arenarius*, and *S. microlepis*. Marx (1959) revised this genus and added other species: *S. dolichospila* and three subspecies for *S. diadema*, including *S. d. diadema*, *S. d. cliffordi*, and *S. d. schiraziana*. Lanza (1964) described *Spalerosophis josephscortecci*, a secretive and endemic species from northwest Somalia. Finally, Schatti et al. (2009, 2010) published a key of the genus *Spalerosophis* according to traditional morphology and discussed the history of the classification of the genus *Spalerosophis*. Until now, six species of *Spalerosophis* are recognized: *S. diadema* from western Sahel to northwest India, *S. arenarius* and *S. atriceps* from north India and Pakistan, *S. dolichospilus* from northwestern Africa, *S. josephscorteccii* from northwest Somalia, and *S. microlepis* from western Iran (Baig & Masroor, 2008; Schatti et al., 2009; Yadollahvandmiandoab et al., 2018).

Most species of *Spalerosophis* are little known, and previous studies have described this genus using only traditional morphology analysis of a few specimens housed in herpetological collections, using material collected decades or even centuries earlier. Some authors have even described some specimens without physical evidence (Schatti et al., 2010). Hence, phylogenetic and evolutionary relationships within this genus have not been assessed and are unresolved. Furthermore, it is unclear whether species and subspecies defined based on morphological and ecological features are well supported by molecular data.

Here, we describe our use of molecular techniques to investigate the phylogenetic relationships, genetic diversity, and divergence time, for four (*S. diadema*, *S. arenarius*, *S. atriceps*, and *S. microlepis*) of the six morphologically recognized species with some doubtful subspecies. To develop an evolutionary hypothesis of their diversification, we examined the DNA sequences from three mitochondrial (cytochrome b, 12S rRNA, and 16S rRNA) genes, and fossil calibrations were used to estimate species divergence times.

Material and methods

Sampling strategy

We gathered 150 tissue samples, mostly muscle of the genus *Spalerosophis* from known localities across the species range from the material deposited in the zoological and natural history museums and collections between 2016 and 2019. Unfortunately, most of the samples were either old or formalin-fixed. DNA extraction from *S. dolichospilus* and *S. josephscorteccii* were unsuccessful due to damaged and low-quality tissue samples. We, therefore, excluded these samples and species from our study and focused on 13 best-preserved samples of other species. Also, 10 GenBank sequences were added to our data. Details of these samples and sequences are summarized in Table 1. Samples were immediately preserved in 99% ethanol and stored in a freezer at − 20 °C for long-term maintenance in the tissue sample collection of the herpetological collection of the Federal University of Paraiba (CHUFPB), until processing.

DNA extraction, PCR, and sequencing

To determine the *Spalerosophis* species' proximity, the whole genomic DNA of 13 specimens were extracted using the Ludwig Blood and Tissue Kit. PCR amplifications were done for part of the 16S gene using the primers L2510mod (5′ CCG ACT GTT TAM CAA AAA CA 3′) and H3056mod (5′ CTC CGG TCT GAA CTC AGA TCA CGT RGG 3′) from Zaher et al. (2009), Cytb gene using the primers L141910 (5′ GAC CTG TGA TMT GAA AAA CCA YCG TTG T 3′) and H16064 (5′ CTT TGG TTT ACA AGA ACA ATG CTT TA 3′) from Burbrink et al. (2000), 12S gene using the primers L1091mod (5′ CAA ACT AGG ATT AGA TAC CCT ACTAT 3′) from Kocher et al. (1989), and H1557mod (5′ GTA CRC TTA CCW TGT TAC GAC TT 3′) from Zaher et al. (2009). Polymerase chain reaction (PCR) conditions for amplification consisted of 1 × buffer, dNTP at 0.2 mM, each primer at 0.2 µM, MgCl2 at 2 mM, 1 U Taq polymerase, and 2 µl of template DNA, in a total reaction volume of 25 µl. PCRs were done with the same conditions for all three genes as follows: 2-min denaturing step at 94 °C followed by 35 cycles of denaturing for 40 s at 94 °C; primer annealing for 45 s at 50 °C for 16S and 50 °C for COI and 12S; and elongation for 1 min at 72 °C, with a final 10 min elongation step at 72 °C. PCR products were loaded onto 1% agarose gel to visualize their quality after electrophoresis. We tried to perform the amplification of the c-mos nuclear gene (CmosS77 and CmosS78 primers from Lawson et al., 2005), but all attempts were unsuccessful. Despite this, previous articles (e.g., Cox et al., 2012; Lawson et al., 2005) indicate a low significant basis of compositional variation among species for nuclear gene for Colubroidea, which supports us to develop the analysis with mitochondrial genes only. The successful amplicons were unidirectionally sequenced in ABI 3130 Genetic Analyzer (Applied Biosystems).

Table 1 Identification and GenBank accession numbers of the *Spalerosophis* samples used in this study

Species	Voucher	Location	Genbank no.			Reference
			16 s	CytB	12 s	
S. diadema schirasianus	01-RZ-01	Iran, Isfahan	ON074602	ON101678	ON074613	This study
S. diadema schirasianus	03-RZ-03	Iran, Yazd	ON074593	-	ON074614	This study
S. diadema schirasianus	05-RZ-05	Iran, Isfahan	ON074598	-	-	This study
S. diadema schirasianus	MHNG 2414.68	Pakistan	-	-	AY039148	Schaetti & Utiger, 2001
S. diadema schirasianus	HSUZM 3265	Iran, Sabzevar	ON074590	-	-	This study
S. diadema schirasianus	HSUZM 1922	Iran, Sabevar	ON074595	ON101679	-	This study
S. diadema cliffordii	SMNH 15,261	Israel, Zofar	ON074599	-	-	This study
S. diadema cliffordii	SMNH 17,784	Israel, Ze'elim	ON074596	ON101680	-	This study
S. diadema cliffordii	SMNH 16,909	Israel, Mizpe Ramon	ON074597	ON101681	-	This study
S. diadema cliffordii	SMNH 17,979	Israel, Nahal Terashim	ON074600	-	ON074615	This study
S. diadema cliffordii	SMNH 15,299	Israel, Nahal Hagav	ON074601	-	ON074616	This study
S. diadema cliffordii	MHNG 2547.44	Yemen, Al Hudaydah	-	-	AY039144	Schaetti & Utiger, 2001
S. diadema cliffordii	Isolate 18	Saudi Arabia, Hail	HQ267787	-	-	Alshammari & El-Abd, 2011, unpublished
S. diadema cliffordii	Isolate 22	Saudi Arabia, Hail	HQ267788	-	HQ658432	Alshammari & El-Abd, 2011, unpublished
S. diadema cliffordii	Isolate 26	Saudi Arabia, Hail	HQ267814	-	HQ658435	Alshammari & El-Abd, 2011, unpublished
S. diadema cliffordii	Isolate 2	Saudi Arabia, Hail	HQ658450	-	HQ658414	Alshammari & El-Abd, 2011, unpublished
S. diadema cliffordii	-	-	KX277269	-	-	Simões et al., 2016
S. diadema cliffordii	ROM 22,879	-	KX694668	KX694865	KX694605	Alencar et al., 2016
S. arenarius	ZFMK 29,290	Pakistan, Karachi	ON074591	-	-	This study
S. atriceps	ZFMK 8092	Pakistan	ON074592	-	-	This study
S. atriceps	ZFMK 86,742	Afghanistan, Mazar-i-Sharif	ON074594	-	ON074617	This study
S. atriceps	USNM 589,837	Afghanistan, Parvan	MG700257	-	-	Gotte et al., 2018, unpublished
S. microlepis	MHNG 2626.70	Iran, Fars	-	-	AY647230	Schatti & Monsch, 2004
Deinagkistrodon acutus	-	Taiwan	KT225463	KT225463	KT225463	Hsieh et al. 2015, unpublished
Protobothrops mangshanensis	-	China	KT963029	KT963029	KT963029	Zhangzhen & Peng, 2015, unpublished
Opisthotropis latouchii	-	China, Zhejiang	MK570292	MK570292	MK570292	Wang et al., 2019
Nerodia sipedon	-	USA	NC_015793	NC_015793	NC_015793	Huff et al. 2011, unpublished

Phylogenetic analyses and species delimitation

We used GENEIOUS v 9.1.3 (Kearse et al., 2012) to check the sequence quality of the strands by comparison to their respective chromatograms and to assemble and edit if necessary. GenBank sequences were also incorporated. Furthermore, nucleotide sequences were aligned using MAFFT v 7.017 (Katoh & Standley, 2013), a module implemented in GENEIOUS v 9.1.3 (Kearse et al., 2012) with default settings. The genetic distances between and within species were estimated to be 16S rRNA and 12S rRNA genes. We adopted the p-distance and computed intraspecific and interspecific p-distance with correspondent standard errors (SE) with 10,000 bootstrap replications and pairwise deletions for gaps using MEGA 11 software (Tamura et al., 2021).

We constructed a multi-gene phylogeny using 16S rRNA, Cytb, and 12S rRNA genes on RAxML (v.8.2.12) (Stamatakis, 2014) and MrBayes 3.2.6 (Ronquist et al., 2012). The maximum-likelihood tree was constructed using the GTR GAMMA model and 1000 bootstrap replicates. The best available model of evolution was selected by jModel Test 2.1.7 (Posada, 2008) (GTR+G). Trees for each gene were also built using this method (see Supplementary files). Two independent parallel runs were run to the Bayesian tree, sampling every 1000th generations for 100 million total generations. The convergence of the parameters was assessed with tracer v.1.6.082. Effective sample sizes (ESS) were

well within acceptable ranges (ESS ≫ 200). After discarding the first 10% of the sampled trees as burn-in, a majority rule consensus tree and posterior probabilities of bipartitions were computed using the remaining trees.

We assessed the taxonomic status of *Spalerosophis* species using three different species delimitations models: (i) Poisson tree processes (PTP), (ii) the multi-rate Poisson tree processes (mPTP), and (iii) the single-threshold method of the generalized mixed Yule-coalescent model (GMYC). We used the complete concatenated dataset. For the PTP and mPTP, a ML tree was reconstructed with RAxML using the GTR GAMMA model of nucleotide substitution and 1000 bootstrap replicates, and the analyses were conducted in the PTP and mPTP web servers (http://species.h-its.org/) using default settings. For the GMYC analysis, an ultrametric Bayesian tree was constructed with BEAST 1.8.4, under the relaxed clock lognormal model and the Yule process speciation model, and the analysis was run for 130 million generations in multiple-locus evaluations. The best sequence evolution models were identified based on their BIC scores with JModeltest v 3.7 (GTR + G). All ESS parameters were higher than 200 (Rambaut et al., 2014). A 10% burn was applied, and the trees were summarized using TreeAnnotator v. 1.8.4. Then, we ran the single-threshold option of GMYC through its web server (http://species.h-its.org/gmyc).

Molecular dating

Molecular dating was based on the three mtDNA (12S, 16S, and Cytb) genes. There are no internal calibration points available for *Spalerosophis*, so we used age estimates based on fossil data for families and division of the Colubridae family. As considered by Daza et al. (2009), based on the oldest colubrid fossil found, it is estimated that the division between Viperidae and Colubridae occurred before 40 MYA (Head et al., 2005; Rage et al., 1992). Thus, a normal distribution of 40 ± 16 MYA was established. We also used the divergence between natricine (Natricinae) from colubrine (Colubrinae) snakes 37.5 ± 7.5 MYA (adapted from Daza et al., 2009; Guicking et al., 2009; Ivanov, 2001; Rage, 1988; Szyndlar, 1991).

To provide these analyses, complete mitochondrial sequences of *Deinagkistrodon acutus* Günther, 1888 (KT225463) and *Protobothrops mangshanensis* Zhao, 1990 (KT963029) for Viperidae and *Nerodia sipedon* Linnaeus, 1758 (NC015793), and *Opisthotropis latouchii* Boulenger, 1899 (MK570292) for Natricinae were included in this analysis as outgroups.

Bayesian estimates of event diversification time were performed in BEAST v1.8.4 program (Drummond & Rambaut, 2007) using a relaxed clock model with a lognormal distribution and other data with standard parameters. For this analysis, we used the concatenated alignment. The best sequence evolution models were identified based on their BIC scores with JModeltest v 3.7 (GTR + G). The analysis was run for 4×10^8 generations with sampling every 10^3 generations. All ESS parameters were higher than 200. A 10% burn was applied to obtain estimates of node age and their respective 95% highest posterior density (HPD). The Tracer v1.6 program (Rambaut et al., 2014) was used to assess the stationarity of the MCMC chain and the 95% HPD range. Trees were summarized using TreeAnnotator v. 1.8.4, choosing "Maximum clade credibility tree" and "Mean heights", which were displayed in FigTree version 1.4.3 (Rambaut, 2017).

Results

Phylogenetic analyses and species delimitation

We used twenty-three specimens for the phylogenetic analyses of the genus *Spalerosophis* (Table 1). The dataset, with a 2278 bp length, included partial sequences of three mitochondrial genes, comprising 16S (544 bp), Cytb (1,090 bp), and 12S (644 bp).

Based on the 16S rRNA sequences, the average uncorrected p-distances between *S. diadema cliffordii* and *S. diadema schirasianus* was 3.72% (SE 0.76%), between *S. diadema cliffordii* and *S. atriceps* was 4.07% (SE 0.80%), and between *S. diadema schirasianus* and *S. atriceps* was 3.45% (SE 0.79%) (see Table 2). Considering 12S rRNA sequences, the average uncorrected p-distances between *S. diadema cliffordii* and *S. diadema schirasianus* was 4.86% (SE 0.99%), between *S. diadema cliffordii* and *S. atriceps* was 4.96% (SE 1.06%), and between *S. diadema schirasianus* and *S. atriceps* was 4.25% (SE 1.14%) (see Table 3).

The phylogenetic trees (Figs. 1 and 2) indicated a monophyletic group (BP = 100%/PP = 100%) with five well-separated species including *S. arenarius*, *S. atriceps*, *S. microlepis*, *S. diadema schirasianus*, and *S. diadema*

Table 2 Genetic distance (in percentage) of the 16S RNA gene fragment between species expressed in values of *p*-distance (below diagonal) and corresponding standard errors (above diagonal) of *Spalerosophis*. Bold values in the main diagonal show the intraspecific genetic distance (mean and standard error). Not computed (n/c) in cases with only one sample for species

	1	2	3	4
1. *S. diadema cliffordii*	**1.39 (0.32)**	0.76	0.88	0.80
2. *S. diadema schirasianus*	3.72	**0.15 (0.11)**	0.95	0.79
3. *S. arenarius*	4.86	5.00	**n/c**	0.75
4. *S. atriceps*	4.07	3.45	0.0309	**0.39 (0.23)**

Table 3 Genetic distance (in percentage) of the 12S RNA gene fragment between species expressed in values of *p*-distance (below diagonal) and corresponding standard errors (above diagonal) of *Spalerosophis*. Bold values in the main diagonal show the intraspecific genetic distance (mean and standard error). Not computed (n/c) in cases with only one sample for species

	1	2	3	4
1. *S. diadema cliffordii*	**0.34 (0.24)**	0.99	1.06	1.16
2. *S. diadema schirasianus*	4.86	**0.10 (0.10)**	0.96	1.14
3. *S. atriceps*	4.96	4.25	**n/c**	1.25
4. *S. microlepis*	5.64	5.57	6.51	**n/c**

cliffordii. Species delimitation analyses (Fig. 1) recognized *S. diadema cliffordii* and *S. diadema schirasianus* as unique and distinct species (67%/95%). The same analyses demonstrated *S. microlepis* as a well-separated species with high support values (100%/100%). Also, two additional species, *S. atriceps* and *S. arenarius*, were separated with good support values (72%/92%).

Molecular dating

According to the BEAST divergence time estimations based on three mtDNA genes, the genus *Spalerosophis* was separated around 35.28 Mya (95% HPD: 25.89–44.66). The Iranian endemic snake, *S. microlepis*, as the first diverged species of the genus, was separated around 21.72 Mya (95% HPD: 10.86–33.04). Then, *S. diadema cliffordii* was separated from *S. diadema schirasianus* about 16.68 Mya (95% HPD: 7.62 -26.62). About 13.31 Mya (95% HPD: 5.52–22.33), *S. arenarius and S. atriceps* diverged from *S. diadema schirasianus*, and finally, further diversification separated *S. arenarius* from *S. atriceps* about 7.87 Mya (95%

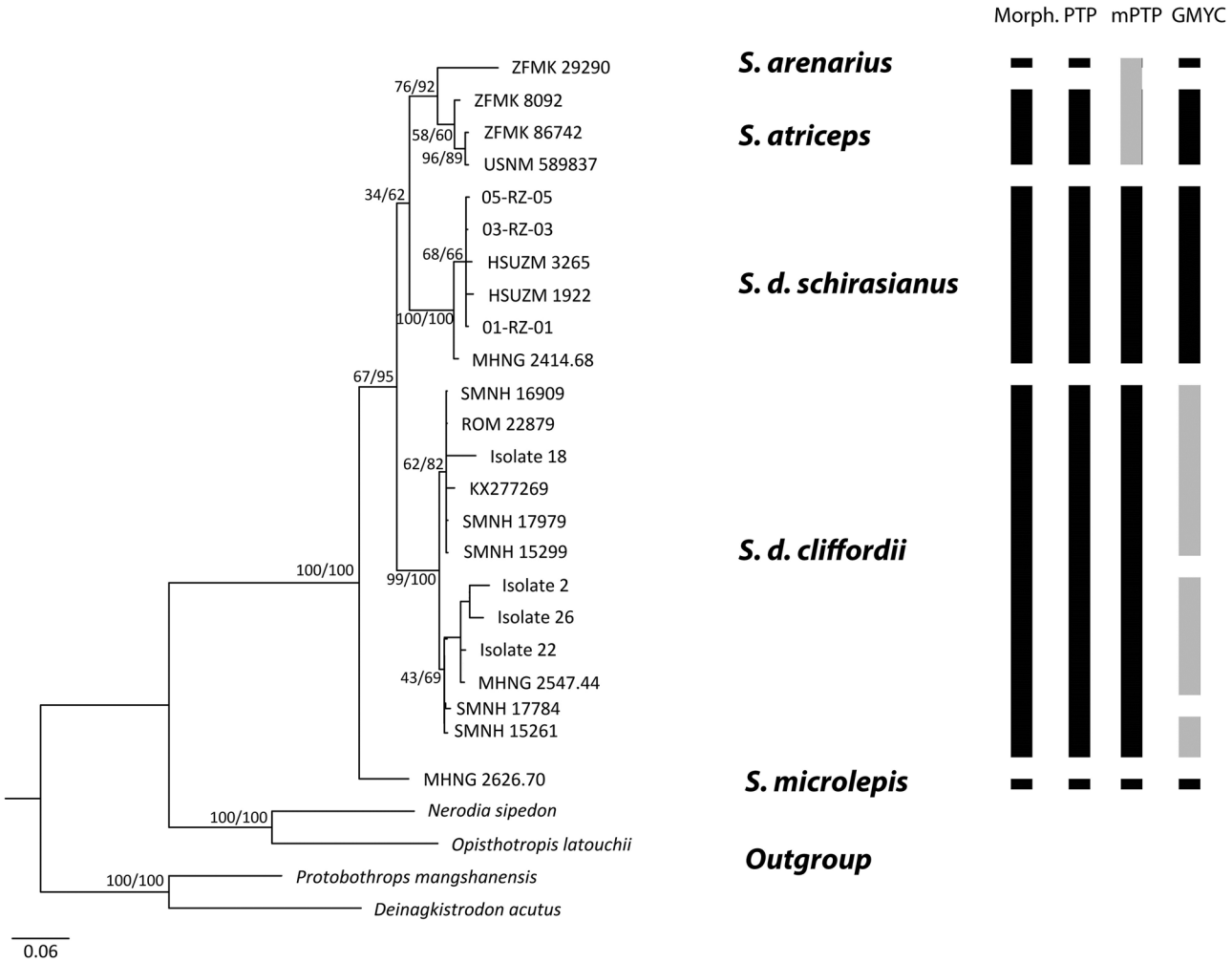

Fig. 1 Bayesian phylogenetic tree of *Spalerosophis* species based on 16 s + Cytb + 12 s genes. Values on the left of nodes denote bootstrap percentage (BP) and Bayesian posterior probabilities in percentage (PP) (BP/PP). Shown on the right are the results of species delimitation analyses using morphological characters (Morph), Poisson tree processes (PTP and mPTP), and general mixed Yule-coalescent (GMYC) analysis

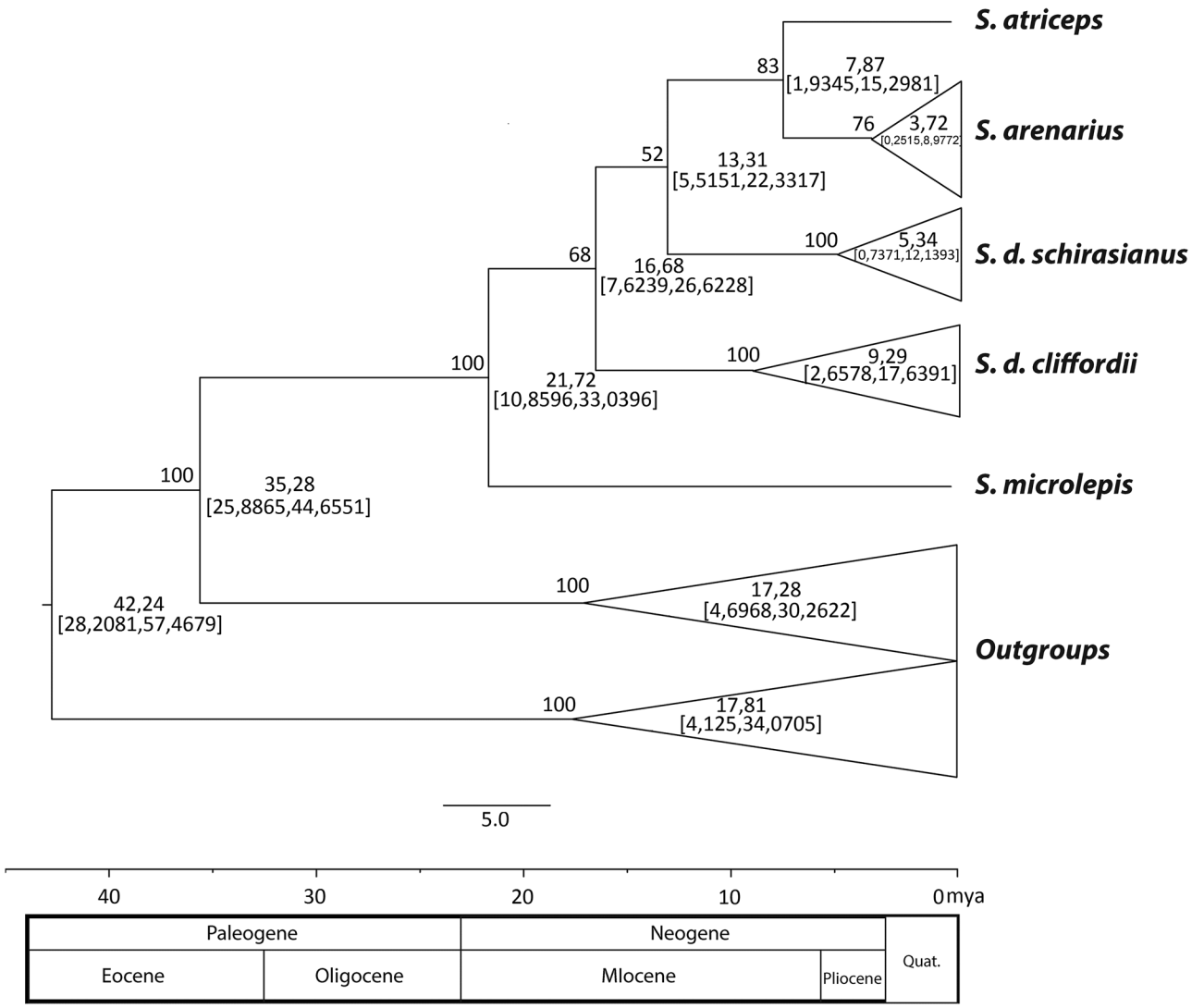

Fig. 2 Divergence times of *Spalerosophis* species based on three mitochondrial genes. Values on the left of nodes denote Bayesian posterior probabilities in percentage, and values on the right of nodes are the estimated median divergence dates with the 95% highest posterior density (HPD)

HPD: 1.93–15.30) (Fig. 2). These ranges belong to the Neogene period extending from 2.58 to 23.03 Mya.

Discussion

Despite the widespread distribution of the genus *Spalerosophis*, there is little information about its systematic phylogenetic structure and evolutionary history. Our study is the first comprehensive molecular investigation of the phylogenetic relationships, diversity, and historical evolution of the genus *Spalerosophis*. Given the long history of taxonomic confusion surrounding the genus, our findings represent a crucial phylogenetic framework for interpreting the latest systematic theories. Although there is much work to arrive at a complete understanding of this complicated genus, we believe that our findings contain sufficient new information to warrant a revision of the *Spalerosophis* taxonomy.

Schatti et al. (2010) divided populations of *S. diadema* into two subspecies just based on the number of subcaudal scales (*S. diadema cliffordii* with < 80 subcaudal scales and *S. diadema schirasianus* with > 80 subcaudal scales), but Marx (1959) described two more subspecies, *S. diadema diadema* with > 100 subcaudal scales and *S. d. dolichospila* recognized based on dorsal oval spots. *S. arenarius* is distinguished from *S. diadema* by the shape and size of its rostral scale plus the coloration of its dorsal surface. *Spalerosophis atriceps* is the largest species of the genus, despite many dissimilarities in morphological characteristics. Marx (1959), Mertens (1969), and Khan (2006) considered *S. atriceps* as a morph and synonym of *S. d.*

diadema. On the other hand, Minton (1966), Baig and Masroor (2008), and Schatti et al. (2009) considered *S. atriceps* as a valid species. Although Marx (1959) introduced the population of *Spalerosophis* snakes in northwestern Africa, as a subspecies of *S. diadema* by the name of *S. d. dolichospila*, Pasteur (1967) did a more comprehensive comparison between this population and the other populations of *S. diadema* and, based on the differences in morphological characteristics and their distributions, raised this northwestern population to species level as *S. dolichospilus* that other authors later adopted (Bons et al., 1996; Schleich et al., 1996). Lanza (1964) described *Spalerosophis josephscorteccii* according to two adult specimens (holotype MZUF 2587 and paratype MZUF 2634) collected from the mountain Oasis of Galgala, Somalia.

If we follow Schatti et al. (2009) and accept *S. dolichospilus* and *S. josephscorteccii*, as separate and unique species, our study, using phylogenetic analyses, confirms the presence of seven species in the genus *Spalerosophis*. Our data indicate that *S. microlepis* was the first diverged species from another species of the genus *Spalerosophis* during the early Miocene (21.72 Mya, 95% HPD: 10.86–33.04). According to Latifi (2000), *S. microlepis* has been reported to occur in mountainous areas, foothills, and grasslands of the Zagros Mountains. Hosseinzadeh et al. (2017) showed that the most suitable habitat for this species lies in relatively humid habitats of mountainous regions, including the Zagros highland that is surrounded on both sides by dry and desert areas. The surrounding dry area is probably the main reason for the long-term isolation of this species in the Zagros Mountains in the west of Iran (Fig. 3).

One interesting and unexpected result of our study is the emergence of two deeply separated species (16S rRNA, mean 3.72%, SE 0.76%; 12S rRNA, mean 4.86%, SE 0.99%), that were previously consistently recognized as subspecies of *S. diadema* solely based on the number of subcaudal scales (Baig & Masroor, 2008; Marx, 1959; Schatti et al., 2009, 2010). The estimated divergence time between *S. d. schirasianus* and *S. d. cliffordii* populations shows that they separated in the late Early Miocene around 16.68 Mya (95% HPD: 7.62–26.62), which coincides with the uplifting of the Zagros Mountains because of the Arabian plate impinging on Eurasia which started ~ 35 Mya (Ghaedi et al., 2021; Salah, 2019). As some previous phylogenetic investigations have shown, the Zagros Mountains act as a natural barrier between the Mesopotamian plain to the west and central Iran and are regarded as the driving force of reptile and amphibian speciation (Fouad & Sissakian, 2011; Ghaedi et al., 2021). These mountains seem to have prevented the gene flow and distribution of *S. diadema* populations between the western and eastern parts. The resulting vicariance events eventually led to allopatric speciation of *S. d. schirasianus* (east of the Mountains) and *S. d. cliffordii* (west of the mountains) (Fig. 3). We thereby strongly endorse separating the two species. This conclusion is also supported by a study on the classification of these two species using traditional morphology and image analysis methods (Yadollahvandmiandoab et al., 2022).

S. arenarius and S. atriceps were recognized as the last diverged members of the genus, separated from *S. d. schirasianus* during the Middle Miocene about 13.31 Mya (95% HPD: 5.52–22.33) and currently inhabit the eastern part of the Sulaiman Mountain in Afghanistan, India, and Pakistan. This kind of distribution can be explained by geological history and vicariance events in these areas. The Sulaiman Mountain ranges were formed by the diagonal collision of the Eurasian and Indo-Pakistan plates in the eastern region of the Iranian Plateau in Afghanistan and Pakistan (Gaina et al., 2015; Ghaedi et al., 2021) (Fig. 3). Although these mountains were uplifted during the Early Miocene, the main complex structural reorganizations occurred during the Late Miocene (Ghaedi et al., 2021; Reynolds et al., 2015; Rodriguez et al., 2014). Unlike Marx (1959), Mertens (1969), and Khan (2006), Yadollahvandmiandoab et al. (2022) using traditional morphology and geometric morphometric analysis showed a high level of differences between *S. atriceps* and *S. diadema* in head shape and morphological characters which corroborated with Minton (1966), Baig and Masroor (2008), and Schatti et al. (2009). It further explained three stages of life of *S. atriceps* with three different colorations that may have caused confusion in the herpetologists in differentiating *S. atriceps* from *S. diadema*. Our phylogenetic study identified *S. atriceps* as a separate and valid species too.

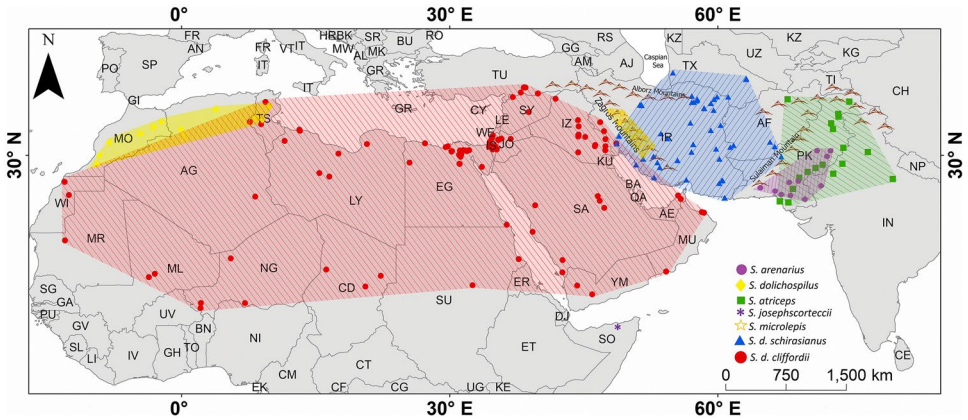

Fig. 3 Geographical distribution range of the genus *Spalerosophis* based on data taken from Marx (1959), Baig and Masroor (2008), www.GBIF.org website, and our study

Our findings corroborate the theory stating that species of *Spalerosophis* originated from an ancestor on the Iranian Plateau and later dispersed to their current distribution zones. The Sulaiman Mountain ranges in the eastern parts of the Iranian Plateau in Afghanistan, India, and Pakistan, and the Zagros Mountains in western Iran that acted as a natural barrier to dispersal may explain the separation of *Spalerosophis* species and their current distribution patterns. Although our phylogenetic analysis provided guidance on adjustments to the taxonomy of *Spalerosophis* snakes at the species level, we suggest that the genetic structures within *Spalerosophis* species and populations be investigated using a more significant number of samples to clarify the genetic variability and phylogeographic patterns of these species. Such an assessment would undoubtedly aid in developing a conservation strategy for this genus.

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Data availability All data is published in the manuscript. Sequences are deposited in Genbank.

Code availability Software and programs are cited in the manuscript.

Declarations

Ethics approval Not applicable.

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References

Alencar, L. R., Quental, T. B., Grazziotin, F. G., Alfaro, M. L., Martins, M., Venzon, M., & Zaher, H. (2016). Diversification in vipers: Phylogenetic relationships, time of divergence and shifts in speciation rates. *Molecular Phylogenetics and Evolution, 105*, 50–62. https://doi.org/10.1016/j.ympev.2016.07.029

Baig, K. J., & Masroor, R. (2008). The snakes of the genus Spalerosophis JAN, 1865 in IndoPakistan and Iran. *Herpetozoa, 20*, 109–115.

Bons, J., Geniez, P., Montori, A., & Roca, V. (1996). Amphibiens et reptiles du Maroc (Sahara occidental compris): atlas biogéographique. *Asociacion herpetologica espanola,* p. 320.

Boulenger, G. A. (1893). *Catalogue of the snakes in the British Museum (Natural History).* Taylor & Francis, p. 73.

Burbrink, F. T., Lawson, R., & Slowinski, J. B. (2000). Mitochondrial DNA phylogeography of the polytypic North American rat snake (Elaphe obsoleta): A critique of the subspecies concept. *Evolution, 54*, 2107–2118. https://doi.org/10.1111/j.0014-3820.2000.tb01253.x

Cox, C. L., Rabosky, A. R. D., Reyes-Velasco, J., Ponce-Campos, P., Smith, E. N., Flores-Villela, O., & Campbell, J. A. (2012). Molecular systematics of the genus Sonora (Squamata: Colubridae) in central and western Mexico. *Systematics and Biodiversity, 10*, 93–108. https://doi.org/10.1080/14772000.2012.666293

Daza, J. M., Smith, E. N., Páez, V. P., & Parkinson, C. L. (2009). Complex evolution in the Neotropics: The origin and diversification of the widespread genus Leptodeira (Serpentes: Colubridae). *Molecular Phylogenetics and Evolution, 53*, 653–667. https://doi.org/10.1016/j.ympev.2009.07.022

Drummond, A. J., & Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology, 7*, 1–8. https://doi.org/10.1186/1471-2148-7-214

Fouad, S. F., & Sissakian, V. K. (2011). Tectonic and structural evolution of the Mesopotamia Plain. *Iraqi Bulletin of Geology and Mining, 4*, 33–46

Gaina, C., Van Hinsbergen, D. J., & Spakman, W. (2015). Tectonic interactions between India and Arabia since the Jurassic reconstructed from marine geophysics, ophiolite geology, and seismic tomography. *Tectonics, 34*, 875–906. https://doi.org/10.1002/2014TC003780

Ghaedi, Z., Badri, S., Saberi-Pirooz, R., Vaissi, S., Javidkar, M., & Ahmadzadeh, F. (2021). The Zagros Mountains acting as a natural barrier to gene flow in the Middle East: More evidence from the evolutionary history of spiny-tailed lizards (Uromasticinae: *Saara*). *Zoological Journal of the Linnean Society, 192*, 1123–1136. https://doi.org/10.1093/zoolinnean/zlaa113

Guicking, D., Joger, U., & Wink, M. (2009). Cryptic diversity in a Eurasian water snake (Natrix tessellata, Serpentes: Colubridae): Evidence from mitochondrial sequence data and nuclear ISSR-PCR fingerprinting. *Organisms Diversity & Evolution, 9*, 201–214. https://doi.org/10.1016/j.ode.2009.03.001

Head, J. J., Holroyd, P. A., Hutchison, J. H., & Ciochon, R. L. (2005). First report of snakes (Serpentes) from the late middle Eocene Pondaung Formation, Myanmar. *Journal of Vertebrate Paleontology, 25*, 246–250. https://doi.org/10.1671/0272-4634(2005)025[0246:FROSSF]2.0.CO;2

Hosseinzadeh, M. S., Ghezellou, P., & Kazemi, S. M. (2017). Predicting the potential distribution of the endemic snake Spalerosophis microlepis (Serpentes: Colubridae), in the Zagros Mountains, western Iran. *Salamandra, 53*, 294–298.

Ivanov, M. (2001). Changes in the composition of the European snake fauna during the Early Miocene and at the Early/Middle Miocene transition. *Paläontologische Zeitschrift, 74*, 563–573. https://doi.org/10.1007/BF02988162

Jan, G. (1865). Prime linee d'una fauna della Persia occidentale (pp. 341–363). In: de FiliPPi, F. Note di un viaggio in Persia nel 1862. Milano, G. Daelli & C. Editori, viii, 396 p.

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution, 30*, 772–780. https://doi.org/10.1093/molbev/mst010

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., & Drummond, A. (2012). Geneious basic: An integrated and extendable desktop software

platform for the organization and analysis of sequence data. *Bioinformatics, 28*, 1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Khan, M. S. (2006). *Amphibians and reptiles of Pakistan* (p. 311). Krieger Publishing Company.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X., & Wilson, A. C. (1989). Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences, 86*, 6196–6200. https://doi.org/10.1073/pnas.86.16.6196

Lanza, B. (1964). Il genere Sphalerosophis e descrizione di una nuova specie (Reptilia, Serpentes). *Monitore Zoologico Italiano, 72*, 47–64.

Latifi, M. (2000). *Snakes of Iran* (p. 478). Department of the Environment.

Lawson, R., Slowinski, J. B., Crother, B. I., & Burbrink, F. T. (2005). Phylogeny of the Colubroidea (Serpentes): New evidence from mitochondrial and nuclear genes. *Molecular phylogenetics and evolution, 37,* 581–601. https://doi.org/10.1016/j.ympev.2005.07.016

Marx, H. (1959). Review of the Colubrid snake genes *Spalerosophis*. *Fieldiana Zoology, 39*, 347–361.

Mertens, R. (1969). Die Amphibien und Reptilien West-Pakistans. *Stuttgarter Beiträge Zur Naturkunde, 197*, 1–96.

Minton, S. A. (1966). A contribution to the herpetology of West Pakistan. *Bulletin of the American Museum of Natural History, 134*, 27–184.

Pasteur, G. (1967). Un serpent endémique du Maghreb: Sphalerosophis [sic] dolichospilus (WERNER), colubridé. *Bulletin Du Muséum National D'histoire Naturelle, Paris, 39*, 444–451.

Posada, D. (2008). jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution, 25*, 1253–1256. https://doi.org/10.1093/molbev/msn083

Rage, J. C. (1988). The oldest known colubrid snakes. The state of the art. *Acta Zoologica Cracoviensia, 31*, 457–474.

Rage, J. C., Buffetaut, E., Buffetaut-Tong, H., Chaimanee, Y., & Ducrocq, S. (1992). A colubrid snake in the late Eocene of Thailand: The oldest known Colubridae (Reptilia, Serpentes). Comptes rendus de l'Académie des sciences. Série 2. *Mécanique, Physique, Chimie, Sciences De L'univers, Sciences De La Terre, 314*, 1085–1089.

Rambaut, A. (2017). FigTree-version 1.4. 3, a graphical viewer of phylogenetic trees. Computer program distributed by the author, website. Retrieved October 5, 2022, from https://www.tree.bio.ed.ac.uk/software/figtree

Rambaut, A., Suchard, M. A., Xie, D., & Drummond, A. J. (2014). Tracer v1. 6.

Reynolds, K., Copley, A., & Hussain, E. (2015). Evolution and dynamics of a fold -thrust belt: The Sulaiman Range of Pakistan. *Geophysical Journal Internationa l, 201*, 683–710. https://doi.org/10.1093/gji/ggv005

Rodriguez, M., Chamot-Rooke, N., Huchon, P., Fournier, M., & Delescluse, M. (2014). The Owen Ridge uplift in the Arabian Sea: Implications for the sedimentary recordof Indian monsoon in Late Miocene. *Earth and Planetary Science Letters, 394*, 1–12. https://doi.org/10.1016/j.epsl.2014.03.011

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A., & Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology, 61*, 539–542. https://doi.org/10.1093/sysbio/sys029

Salah, M. (2019). Seismological evidence for lithospheric low-velocity anomalies beneath the eastern Mediterranean: Impact of tectonics. *Geotectonics, 53*, 617–633. https://doi.org/10.1134/S0016852119050054

Schatti, B., Tillack, F., & Helfenberger, N. (2009). A contribution to Spalerosophis microlepis Jan 1865, with a short review of the genus and a key to the species (Squamata: Serpentes: Colubridae). *Herpetozoa, 22*, 115–135.

Schatti, B., & Utiger, U. (2001). Hemerophis, a new genus for Zamenis socotrae Günther, and a contribution to the phylogeny of Old World racers, whip snakes, and related genera (Reptilia: Squamata: Colubrinae). *Revue Suisse De Zoologie, 108*, 919–948.

Schatti, B., Ineich, I., & Kucharzewski, C. (2010). Nominal taxa of Spalerosophis diadema (Schlegel, 1837) from Iraq to Pakistan - two centuries of confusion (Reptilia: Squamata: Colubrinae). *Revue Suisse De Zoologie, 117*, 637–664.

Schatti, B., & Monsch, P. (2004). Systematics and phylogenetic relationships of whip snakes (Hierophis Fitzinger) and Zamenis andreana Werner, 1917 (Reptilia: Squamata: Colubrinae). *Revue Suisse De Zoologie, 111*, 239–256.

Schleich, H. H., Kästle, W., & Kabisch, K. (1996). *Amphibians and reptiles of North Africa*. Koeltz scientific, Koenigstein, p. 627.

Schmidt, K. P. (1930). Reptiles of Marshall Field North Arabian desert expeditions 1927–1928. *Field Museum of Natural History, Zoology Series, 17*, 223–230.

Simões, B. F., Sampaio, F. L., Douglas, R. H., Kodandaramaiah, U., Casewell, N. R., Harrison, R. A., Hart, N. S., Partridge, J. C., Hunt, D. M., & Gower, D. J. (2016). Visual pigments, ocular filters and the evolution of snake vision. *Molecular Biology and Evolution, 33*, 2483–2495. https://doi.org/10.1093/molbev/msw148

Stamatakis, A. (2014). RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, *30,* 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Szyndlar, Z. (1991). A review of Neogene and Quaternary snakes of central and eastern Europe. Part 1: Scolecophidia, Boidae. *Colubrinae. Estudios Geológicos, 47*, 103–126. https://doi.org/10.3989/egeol.91471-2412

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution, 38*, 3022–3027. https://doi.org/10.1093/molbev/msab120

Uetz, P., Freed, P., Aguilar, R., & Hošek, J. (2021). The Reptile Database. Retrieved October 10, 2021, from https://www.reptledatabase.org/

Wang, Y., Liu, P., Li, H., & Shao, C. (2019). The complete mitochondrial genome of Opisthotropis latouchii (Squamata: Colubridae). *Mitochondrial DNA Part B, 4*, 1437–1438. https://doi.org/10.1080/23802359.2019.1598816

Yadollahvandmiandoab, R., Bashirichelkasari, N., & Oliveira Mesquita, D. (2022). Morphological characterization of some poorly known populations of the genus *Spalerosophis* Jan, 1865, (Reptilia: Colubridae): Is the traditional morphology and geometric morphometrics useful to identify them as populations?. *Zoology in the Middle East*, (in review).

Yadollahvandmiandoab, R., Oliveira Mesquita, D., & Kami, H. G. (2018). A preliminary study on the biology of the Diadem snake (Spalerosophis diadema), from Iran (Reptilia: Colubridae). *Herpetology Notes, 11*, 481–487.

Zaher, H., Grazziotin, F. G., Cadle, J. E., Murphy, R. W., Moura-Leite, J. C. D., & Bonatto, S. L. (2009). Molecular phylogeny of advanced snakes (Serpentes, Caenophidia) with an emphasis on South American Xenodontines: A revised classification and descriptions of new taxa. *Papéis Avulsos De Zoologia, 49*, 115–153.