



Genetic Structure of the Spider-Tailed Viper, *Pseudocerastes urarachnoides* (Ophidia, Viperidae), in Western Iran Inferred from Mitochondrial DNA Sequences

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Abstract Understanding the genetic structure of a population is essential for the effective conservation of threatened species. This study presents the first analysis of the population genetic structure of the spider-tailed viper (*Pseudocerastes urarachnoides*) in western Iran, based on *Cytb* and *CR1* mitochondrial gene sequences. Using spatial analysis of molecular variance and principal coordinates analysis, we delineated five distinct populations. We observed a pattern of low nucleotide diversity (π) and high haplotype diversity (H_d), with significant genetic differentiation between populations ($\phi_{ST} = 0.1-0.92$, $P < 0.05$). Intra-population genetic diversity was significantly lower than inter-population diversity ($P < 0.001$). The haplotype network analysis confirmed the existence of five distinct haplogroups for *Cytb*, and four for *CR1* and the combined dataset. This reveals a general lack of genetic connectivity between these populations. Although neutrality tests (Tajima's D , Fu's

F_s) and the raggedness index suggested population stability, a pattern of isolation by distance was evident across the species' range. These results define five key management units for *P. urarachnoides*. As a unique endemic species facing significant threats, these findings provide the Iranian Department of Environment with a critical genetic foundation on which to develop targeted conservation strategies to preserve this viper and its associated habitats.

Keywords conservation genetics, F_{ST} , Management units, population structure, *Pseudocerastes urarachnoides*, Tajima's D , west of Iran

1. Introduction

Anthropogenic threats, including habitat destruction, climate change, and overexploitation, are driving a global biodiversity crisis and placing an estimated one million species at risk of extinction (IPBES, 2019). These external pressures are often exacerbated by concomitant declines in genetic diversity. Population decline and fragmentation can increase inbreeding, thereby reducing genetic diversity, which ultimately diminishes individual fitness and a population's capacity to adapt (Ceballos *et al.*, 2017). Therefore, population genetic studies are critical for understanding and mitigating threats to wildlife (Hohenlohe *et al.*, 2021).

A species' population structure determines its evolutionary potential and is shaped by forces such as gene flow, genetic drift, and selection. Understanding the historical and contemporary impacts of these forces is essential for

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Received: 4 June 2025 Accepted: 30 March 2026

Published Online: 3 April 2026

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interpreting current genetic patterns (Slatkin, 1987; Garnier *et al.*, 2004). In small populations, genetic drift can trigger an extinction vortex, where loss of diversity leads to inbreeding depression and reduced fitness, further accelerating population decline (Gilpin, 1986; Lynch *et al.*, 1995). Therefore, accurate quantification of population structure and spatial patterns of genetic diversity is a cornerstone of conservation biology.

Spatial genetic patterns are observed as two distinct patterns. Isolation-by-distance (IBD) occurs when genetic differentiation between individuals increases with geographic distance due to limited dispersal (Wright, 1943). In contrast, isolation-by-environment (IBE) arises when adaptive divergence or selective barriers reduce gene flow between different habitats, independent of geographic distance (Wang and Bradburd, 2014).

The spider-tailed viper (*Pseudocerastes urarachnoides*), which is endemic to the Zagros Mountains, is under threat from habitat loss and illegal collection for the pet trade. It is listed in CITES Appendix II and classified as Near Threatened on the IUCN Red List (Mobaraki *et al.*, 2022). Despite studies on its ecology and venom (Fathinia *et al.*, 2009; Fathinia and Rastegar-Pouyani, 2010; Fathinia, 2014; Fathinia *et al.*, 2014; Fathinia *et al.*, 2015; Fathinia *et al.*, 2017; Fathinia *et al.*, 2018; Fathinia *et al.*, 2020; Ghezellou *et al.*, 2021; Op den Brouw *et al.*, 2021; Akbarpour *et al.*, 2023), the population genetic structure and genetic diversity remain completely unknown, hindering effective, science-based conservation efforts.

This study provides the first population genetic assessment of the spider-tailed viper using mitochondrial DNA. We aim to: (a) quantify genetic diversity within and among populations; (b) estimate effective population sizes; (c) identify populations of the highest and lowest conservation priority; and (d) elucidate relationships between populations through haplotype analysis.

2. Materials and Methods

2.1. Study area, samples, and laboratory procedures During a 10-year field study from 2012 to 2022, 46 specimens of *P. urarachnoides* were collected from western Iran, mainly in Ilam (including Abdanan, Mehran, Malekshahi, Ilam, and Chavar townships) and Kermanshah (Qasr-e-Shirin township)

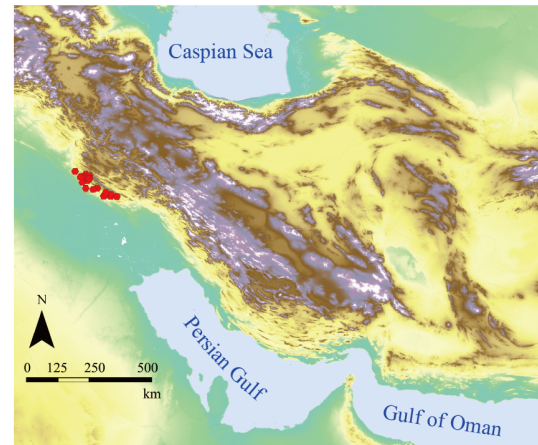


Figure 1 Geographic distribution of samples (red hexagons) collected in western Iran.

provinces (Figure 1). The coordinates of the samples were recorded using a Garmin GPS device or a smartphone. Tissue samples were obtained from blood via venipuncture or cardiocentesis (Köbölkuti *et al.*, 2009) or from exuvia and preserved in 96% or absolute ethanol. To extract total genomic DNA, we used a SinaPure DNA extraction kit (EX6001; <https://sinaclon.ir/>). Partial segments of the mitochondrial genes cytochrome b (*Cytb*) and control region 1 (*CR1*) were amplified, sequenced, and used in subsequent population genetic analyses. We used PCR to amplify these loci with a total volume of 25 μ L PCR reactions (10 μ L PCR mix) and the following volumes or reagents: 1 μ L forward primer, 1 μ L reverse primer, 11 μ L ultrapure water, and 2 μ L template DNA. Supplementary Table S1 provides details on the primers used and PCR conditions.

2.2. Data analyses We used Chromas v. 2.6.6 to assess sequence quality, trim low-quality ends, and convert the sequences to FASTA format. *Cytb* and *CR1* sequences were aligned with the online version of MAFFT using FFT-NSi and G-INS-1 (VSM-amax = 0.8), respectively (Katoh *et al.*, 2019).

2.3. Haplotype network inference Population analysis with reticulate trees (PopART) is a software package that performs population genetic analyses using haplotype networks. Three methods—minimum spanning, median-

Table S1 Data on primer sequences, primer sources, and PCR programs.

Gene	Primer	Primer sequence	PCR program	Source
<i>Cytb</i>	L14919 (F)	5'-AACCACCGTTGTTATTCAACT-3'	94°(4); 94°(45°); 50°(45°); 72°(90°); 72°(10°)	(Rastegar-Pouyani <i>et al.</i> , 2010)
	Ei700R (R)	5'-GGGGTGAAAGGGGATTTTRTC-3'		
<i>CR1</i>	L16571VA (F)	5'-CTCTTTCCAAGGCCTCTGGCT-3'	95°(3); 95°(60°); 50°(60°); 72°(60°); 72°(5°)	(Kumazawa <i>et al.</i> , 1996; Kumazawa <i>et al.</i> , 1998)
	H690 (R)	5'-GTTGAGGCTTGCATGTATA-3'		

joining, and TCS network—are applied in PopART (Leigh and Bryant, 2015). PopART v. 1.7 (Leigh and Bryant, 2015) was used to generate a TCS haplotype network (Clement *et al.*, 2002) and to investigate haplotype patterns within *P. urarachnoides* across its distribution. This analysis was conducted on each locus separately and on a concatenated alignment of the two loci.

2.4. Population genetic analyses Grouping of samples into discrete populations was conducted using spatial analysis of molecular variance (SAMOVA) implemented in Arlequin v. 3.5 (Excoffier and Lischer, 2010) and principal coordinates analysis (PCoA) in GenAlex v. 6.5 (Peakall and Smouse, 2012). These groupings form the basis for subsequent population genetic analyses.

Haplotype diversity (H_d) and nucleotide diversity (π) are two key parameters used in assessing population diversity and genetic health (Nei, 1987; Gollner *et al.*, 2016). Haplotype diversity ranges from 0 (indicating one fixed haplotype among all samples) to 1 (indicating a different haplotype for each sampled individual). The π index calculates the likelihood of two different nucleotides occurring at a specific point in gene sequences when two samples are randomly compared (Gollner *et al.*, 2016). We used DnaSP v. 5 (Librado and Rozas, 2009) to estimate N_h (number of haplotypes), H_d (haplotype diversity), and π (nucleotide diversity). We ran an analysis of molecular variance (AMOVA) to examine population genetic structure. Pairwise and overall F_{ST} statistics were computed using the AMOVA test with 10 000 permutations in Arlequin v. 3.5 (Excoffier and Lischer, 2010). According to the classification by Balloux and Lugon-Moulin (2002), population differentiation based on F_{ST} is divided into four levels: low (0–0.05), medium (0.05–0.15), high (0.15–0.25), and very high (> 0.25).

To determine the degree of gene flow (N_m) among populations, we used DnaSP (Librado and Rozas, 2009), with 1 000 random replicates. This index reflects average population size (N) and the ratio of immigrants (m), indicating the degree to which each local population functions as an independent evolutionary unit (Dutta *et al.*, 2020).

To investigate whether the spider-tailed viper populations have experienced historical population growth, a mismatch distribution pattern analysis (Rogers and Harpending, 1992; Excoffier, 2004) was performed. Demographic history indices, such as Tajima's D , Fu's F_s , R_2 , and the raggedness index (rg), used to test for population expansion or contraction, were calculated in DnaSP v. 5. It is recommended that all four parameters be analyzed in parallel, as each has its own sensitivities to population size changes or selection (Gollner *et al.*, 2016). The indices Tajima's D (Tajima, 1989), Fu's F_s (Fu,

1997), and R_2 (Ramos-Onsins and Rozas, 2002) are used to detect the presence of anomalous frequencies of singleton mutations in populations that occur with neutral evolution when a population is out of mutation-drift equilibrium due to rapid expansion. The rg index (Harpending *et al.*, 1993) is also expected to show a unimodal pattern under conditions of population expansion, as it reflects the distribution of mismatches in pairwise nucleotide differences among haplotypes. In this framework, the observed distribution was compared with the distribution predicted under the sudden expansion model (Rogers and Harpending, 1992; Rogers, 1995), and goodness-of-fit tests (Schneider and Excoffier, 1999) were used to assess model fit. A smooth unimodal mismatch distribution is indicative of sudden population expansion or decline, whereas a more gently sloping pattern is more consistent with a stable population without drastic changes. The indicators of population expansion (i.e., τ , θ_0 , and θ_1) were calculated in Arlequin (Excoffier *et al.*, 2005), with both loci combined. To estimate the time of population expansion (t), we used the equation $t = \tau/2u$, where u is the cumulative probability of substitution over the length of the sequence, differing from μ , the substitution rate per nucleotide (Schenekar and Weiss, 2011). The average mitochondrial substitution rate in snakes and lizards, 5.29×10^9 , was used for this calculation (Eo and DeWoody, 2010). The indices r (raggedness) and SSD (sum of squared deviations) were computed for each population in Arlequin, with non-significant results indicating population expansion (Dutta *et al.*, 2020).

The ratio of nonsynonymous/synonymous substitutions ($\omega = K_a/K_s$) is a tool for detecting the type of selective pressure on a gene, such that $\omega \approx 1$ indicates neutral selection, $\omega < 1$ indicates purifying or negative selection, and $\omega > 1$ indicates positive or adaptive selection (Zhang, 2022). To assess the significance of the difference between K_a and K_s , a codon-based Z-test was also used, and its variance was estimated using the bootstrap method, with 1 000 replications in MEGA-X (Kumar *et al.*, 2018).

Lastly, we ran a Mantel test in Arlequin with 10 000 permutations to evaluate correlations between genetic and geographic distances at both intra- and interpopulation levels, revealing the extent of IBD within the distribution of this species.

3. Results

3.1. Mitochondrial DNA diversity A total of 44 sequences of the partial *Cytb* gene (583 bp) and 46 sequences of the *CR1* gene (536 bp) were generated. For any analyses using both loci, only 44 sequences of the concatenated data were used; this concatenated alignment

was 1 120 bp in length (hereafter referred to as combined data). The sequences were deposited in NCBI under the codes PX987023 to PX987066 for *Cytb*, and PX987067 to PX987112 for *CR1*. Genetic diversity included 43, 96, and 136 polymorphic sites, as well as 25, 36, and 39 haplotypes in the *Cytb*, *CR1*, and combined data, respectively. The high number of polymorphic sites and haplotypes suggests these mitochondrial genome fragments are suitable for population genetic analyses among the spider-tailed viper populations. The average nucleotide composition of the partial *Cytb* gene is 27.27% A, 32.94% C, 13.52% G, and 26.28% T. For *CR1*, it is 27.43% A, 28.50% C, 9.63% G, and 34.44% T. The nucleotide composition of the combined data is 27.35% A, 30.84% C, 11.68% G, and 30.13% T.

3.2. Population genetic structure

Spatial genetic variation Analysis of SAMOVA identified five populations of *P. urarachnoides* in western Iran (fixation index representing the proportion of genetic variation among groups of populations relative to the total variation, or $F_{CT} = 0.71$; $P = 0.0001$), with population sizes ranging from 3 to 13 sampled individuals (Figure 2). Similarly, PCoA based on pairwise genetic distances revealed five populations within three distinct clusters (Figure 3). The composition of individuals in these five populations from the two different analyses was in agreement. Eigenvalues for PC1 and PC2 are 866.5 and 145.25, respectively, accounting for 83.9% and 14.06% of the total variance. Both SAMOVA and PCoA analyses support dividing the dataset into five populations, hereafter referred to as populations 1 to 5 (Figure 2).

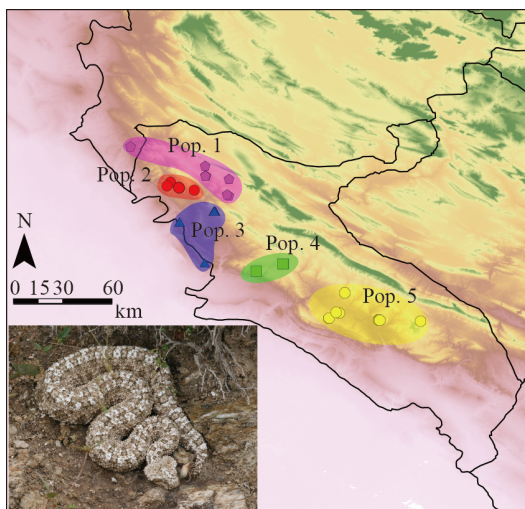


Figure 2 Map of five distinct populations of the spider-tailed viper in western Iran, as suggested by SAMOVA and PCoA analyses (with 9, 13, 9, 3, and 10 individuals in populations 1 to 5, respectively).

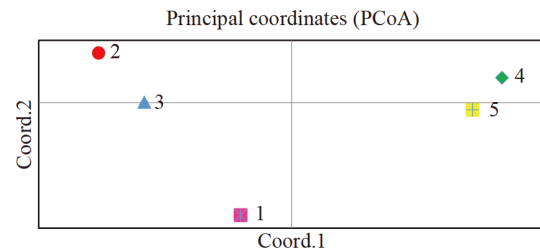


Figure 3 PCoA analysis using pairwise distances revealed five distinct populations of the spider-tailed viper within three clusters in western Iran. Coord. = Coordination.

Nucleotide, haplotype, and genetic diversity Nucleotide diversity (π) of the partial *Cytb* gene varies from 0.0025 (population 1) to 0.0051 (population 2), while Hd ranges from 0.8 (population 5) to 1 (population 4). For the *CR1* locus, π ranges from 0.0004 (population 1) to 0.05 (population 2), and Hd from 0.22 (population 1) to 1.00 (populations 2 and 4). In the combined data, π varies from 0.0015 (population 1) to 0.023 (population 2), and Hd from 0.83 (population 1) to 1.00 (populations 2, 4, and 5) (Table 1). Nucleotide and haplotype diversity indices across each gene and the combined data are similar for each population, except in population 1, where both measures are notably lower for *CR1* (Table 1).

AMOVA results indicate that for *Cytb*, genetic diversity is mainly due to intra- and interpopulation variation at rates of 19.25% and 80.75%, respectively ($P = 0.0001$). For *CR1*, intra- and interpopulation variation account for 41.84% and 58.16%, respectively ($P = 0.0001$), and for the combined data, these are 32.76% and 67.24% ($P = 0.0001$). Interpopulation F_{ST} values indicate significant differences, ranging from low to high: from 0.04 to 0.18 for *Cytb*, up to 0.56 for *CR1*, and from low to medium at about 0.11 for the concatenated data (Table 2). Significant differences in F_{ST} were observed only among specific populations (Table 2). The Φ_{ST} index shows moderate to very high differences ranging from 0.3 to 0.94 ($P < 0.05$) in partial *Cytb*, from 0.09 to 0.94 ($P < 0.05$) in *CR1* (except for population pair 2 vs. 3), and 0.1 to 0.92 ($P < 0.05$) for the concatenated data (Table 2).

Gene flow and genetic differentiation Gene flow (N_m) among populations ranges from low to medium levels (Table 3). N_m values above 1 indicate substantial gene flow that counteracts genetic differentiation, while higher Φ_{ST} paired with lower N_m values highlight greater differentiation, especially between populations 2 and 3, which show significant differentiation with minimal gene flow.

Demographic history Haplotype networks using the TCS method revealed distinct haplogroups in *Cytb*, *CR1*, and the

Table 1 Nucleotide and haplotype diversity among different populations of the spider-tailed viper in western Iran. Abbreviations: No., number of individuals; π , nucleotide diversity; Nh, number of haplotypes; Hd, haplotype diversity; SD, standard deviation; Comb., combined data. Ka/Ks is the ratio of non-synonymous to synonymous substitutions in the protein-coding *Cytb* gene. The significance value (P) was obtained from the codon-based Z-test.

Population	Gene	No.	$\pi \pm SD$	Nh	Hd $\pm$ SD	Ka/Ks (P)
1	<i>Cytb</i>	9	0.0025 $\pm$ 0.0006	5	0.83 $\pm$ 0.009	(0.035) 0.000
	<i>CR1</i>	9	0.0004 $\pm$ 0.0003	2	0.22 $\pm$ 0.16	–
	Comb.	9	0.0015 $\pm$ 0.0004	5	0.83 $\pm$ 0.098	–
2	<i>Cytb</i>	13	0.0051 $\pm$ 0.0018	9	0.936 $\pm$ 0.05	(0.048) 0.152
	<i>CR1</i>	14	0.05 $\pm$ 0.007	14	1.00 $\pm$ 0.027	–
	Comb.	13	0.023 $\pm$ 0.004	13	1.00 $\pm$ 0.03	–
3	<i>Cytb</i>	9	0.0038 $\pm$ 0.001	5	0.833 $\pm$ 0.09	(0.241) 0.000
	<i>CR1</i>	9	0.022 $\pm$ 0.005	8	0.97 $\pm$ 0.06	–
	Comb.	9	0.012 $\pm$ 0.0024	8	0.972 $\pm$ 0.064	–
4	<i>Cytb</i>	3	0.0046 $\pm$ 0.0017	3	1.00 $\pm$ 0.27	(0.108) 0.107
	<i>CR1</i>	3	0.01 $\pm$ 0.003	3	1.00 $\pm$ 0.27	–
	Comb.	3	0.007 $\pm$ 0.002	3	1.00 $\pm$ 0.272	–
5	<i>Cytb</i>	10	0.0044 $\pm$ 0.0017	5	0.8 $\pm$ 0.1	(0.267) 0.214
	<i>CR1</i>	11	0.005 $\pm$ 0.0009	9	0.96 $\pm$ 0.05	–
	Comb.	10	0.004 $\pm$ 0.0008	10	1.00 $\pm$ 0.045	–
Populations combined	<i>Cytb</i>	44	0.02 $\pm$ 0.0012	25	0.97 $\pm$ 0.01	(0.427) 0.074
	<i>CR1</i>	46	0.04 $\pm$ 0.004	36	0.97 $\pm$ 0.018	–
	Comb.	44	0.027 $\pm$ 0.002	39	0.993 $\pm$ 0.007	–

Table 2 Estimated values of Φ_{ST} (regular numbers above the grey diagonal) and F_{ST} (italic numbers below the diagonal) for *Cytb*, *CR1*, and the combined data among all pairs of populations of the spider-tailed viper in western Iran. An asterisk indicates significant differences ($P < 0.05$) between population pairs, obtained after 10 000 permutations. Comb. = combined data.

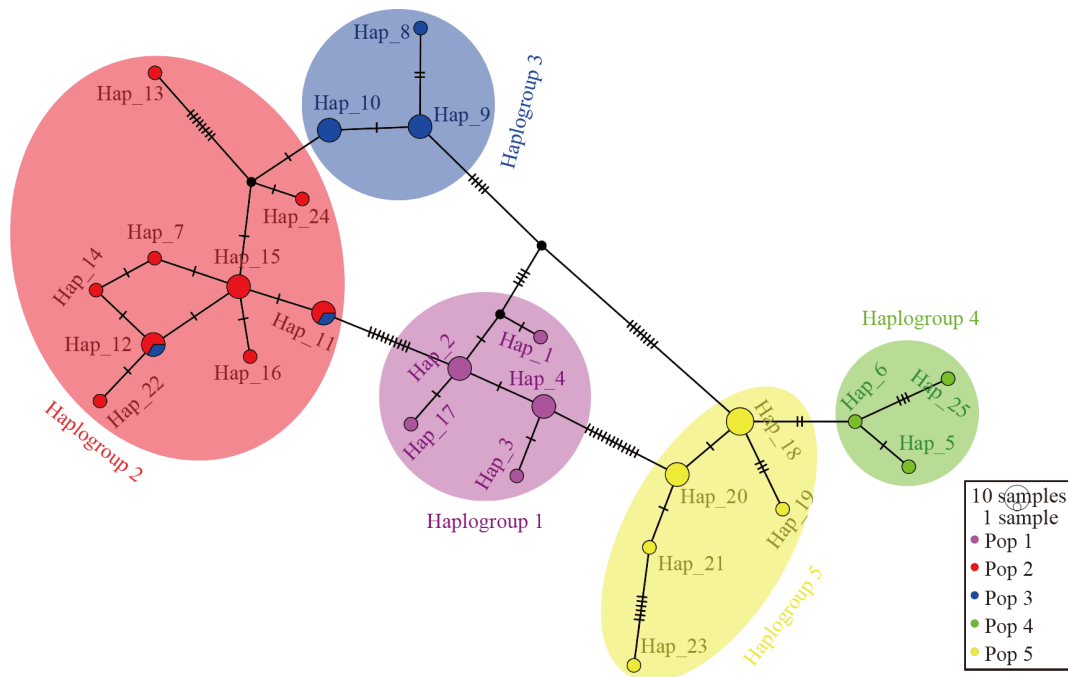
Population	1	2	3	4	5
1	<i>Cytb</i>	0.86*	0.88*	0.94*	0.91*
	<i>CR1</i>	0.45*	0.45*	0.45*	0.45*
	Comb.	0.59*	0.72*	0.92*	0.89*
2	0.11*	<i>Cytb</i>	0.3*	0.93*	0.92*
	0.35*	<i>CR1</i>	0.09	0.43*	0.57*
	0.08*	Comb.	0.1*	0.61*	0.68*
3	0.17*	0.08*	<i>Cytb</i>	0.94*	0.93*
	0.4*	0.01	<i>CR1</i>	0.63*	0.72*
	0.1*	0.01	Comb.	0.74*	0.78*
4	0.11	0.04	0.11	<i>Cytb</i>	0.45*
	0.56*	0.00	0.02	<i>CR1</i>	0.30*
	0.11	0.00	0.02	Comb.	0.37*
5	0.18*	0.13*	0.18*	0.13	<i>Cytb</i>
	0.39*	0.02*	0.03	0.02	<i>CR1</i>
	0.08*	0.00	0.01	0.00	Comb.

combined data (Figures 4–6). The *Cytb* haplotype network aligns with the groupings from SAMOVA, but in *CR1* and the combined data, haplogroups 2 and 3 intermingle and contain the highest number of haplotypes (Table 1). The most common haplotype in *Cytb* is Hap_18, found four times in

population 5 (Figure 4); in *CR1*, Hap_1 appears eight times in population 1; and in the combined data, Hap_2 and Hap_4 each appear three times in population 1 (Figure 6). Haplotypes Hap_11 and Hap_12 in *Cytb* are shared between haplogroups 2 and 3 (Figure 4). No shared haplotypes are observed among

Table 3 Estimated values of the Nm index based on 10 000 permutations for *Cytb*, *CR1*, and the combined data across populations of the spider-tailed viper in western Iran. Abbreviations: Comb. = combined data.

Population	1	2	3	4	5
2	0.11	<i>Cytb</i>			
	0.50	<i>CR1</i>			
	0.30	Comb.			
3	0.09	1.2	<i>Cytb</i>		
	0.33	6.43	<i>CR1</i>		
	0.21	4.84	Comb.		
4	0.07	0.08	0.07	<i>Cytb</i>	
	0.07	0.41	0.26	<i>CR1</i>	
	0.07	0.24	0.16	Comb.	
5	0.08	0.09	0.08	0.69	<i>Cytb</i>
	0.05	0.37	0.23	1.28	<i>CR1</i>
	0.06	0.23	0.15	0.91	Comb.

**Figure 4** Mitochondrial *Cytb* TCS haplotype network of the spider-tailed viper in western Iran. Distinct haplotypes are represented as monochromatic circles (indicating haplotypes found only in one location), while shared haplotypes (found in more than one location) are represented as dichromatic circles with diameters proportional to their frequency. Black dots and slashes indicate missing haplotypes (providing a relative estimate of genetic divergence between haplotypes). Hap = Haplotype; Pop = Population.

haplogroups in *CR1* and the combined data, where all haplotypes are private (Figures 5–6). In these networks, populations 2 and 3 form a shared haplogroup, while populations 1, 4, and 5 are distinct. Haplogroup 1 in both *Cytb* and the combined network serves as an intermediate between haplogroups 2 and 3, and those of 4 and 5, while this pattern is not seen in *CR1*. Variations among haplotypes

within each haplogroup—marked by small lines perpendicular to connecting lines—are generally minor but higher between haplogroups.

Due to the small sample size, it was not possible to calculate Fu's F_s and Tajima's D for population 4. Fu's F_s results vary between populations: with non-significant values in populations 1 and 3, but significant values in populations 2

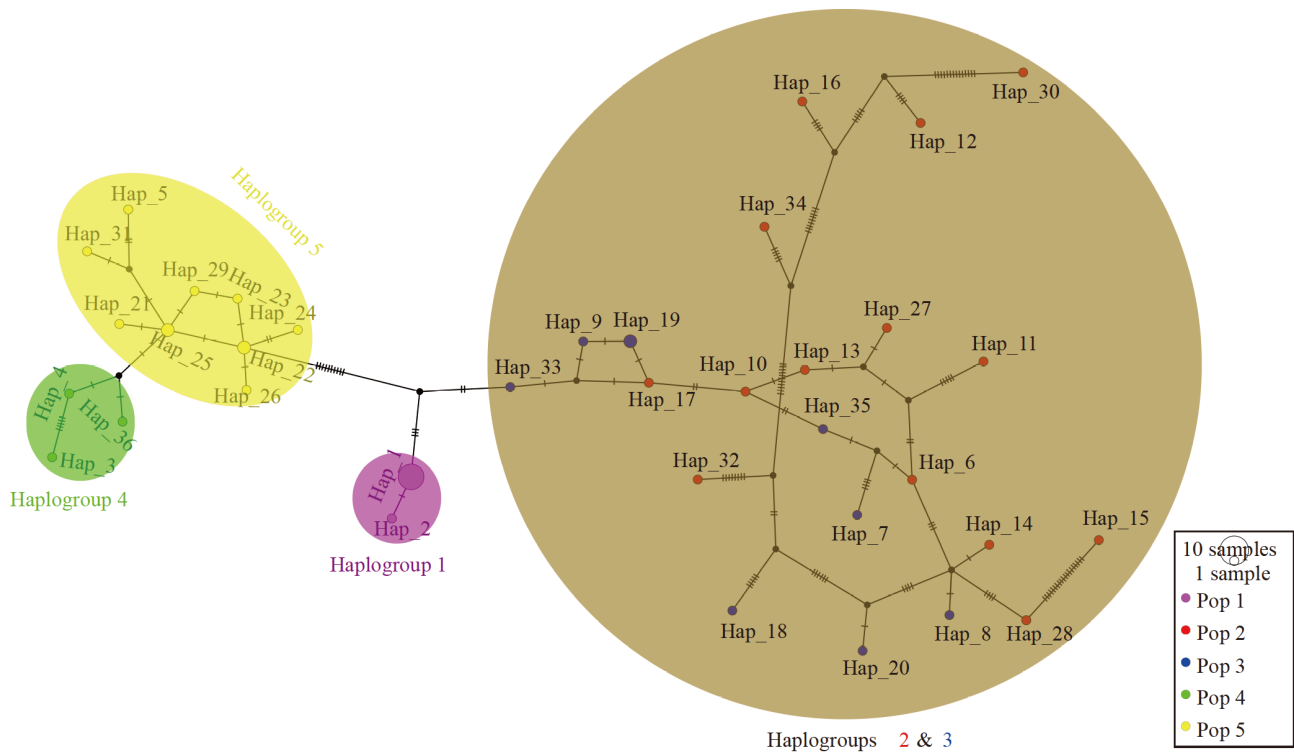


Figure 5 Mitochondrial *CR1* TCS haplotype network of the spider-tailed viper in western Iran. Distinct haplotypes are represented as monochromatic circles (indicating haplotypes found only in one location) with diameters proportional to their frequency. Black dots and slashes indicate missing haplotypes (providing a relative estimate of genetic divergence between haplotypes). Hap = Haplotype; Pop = Population.

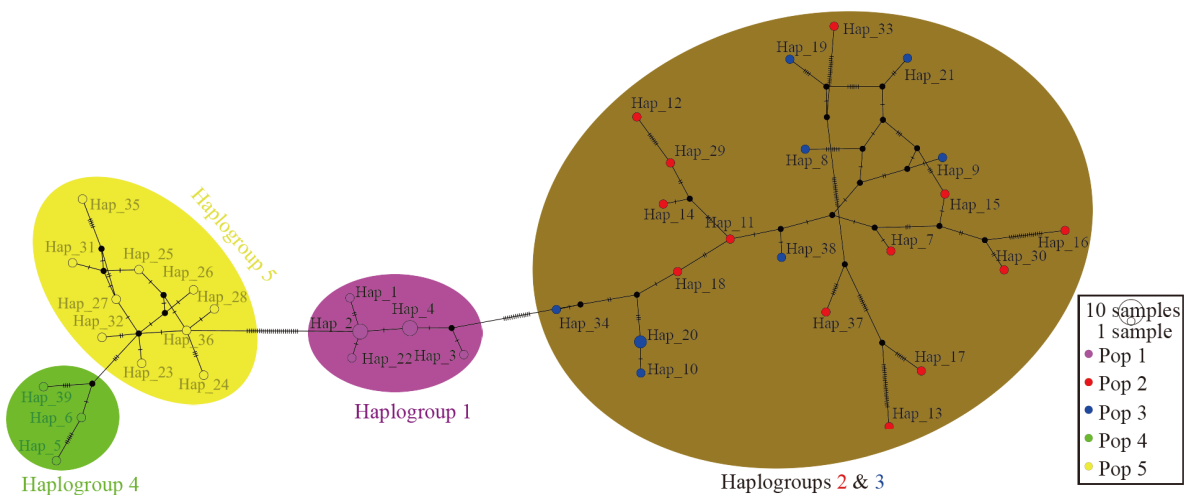


Figure 6 Mitochondrial combined data TCS haplotype network of the spider-tailed viper in western Iran. Distinct haplotypes are represented as monochromatic circles (indicating haplotypes found only in one location) with diameters proportional to their frequency. Black dots and slashes indicate missing haplotypes (providing a relative estimate of genetic divergence between haplotypes). Hap = Haplotype; Pop = Population.

and 5. The overall F_u 's F_s for all populations combined is negative and significant. Tajima's D is negative across the population for *Cytb* (−0.61 to −1.58), *CR1* (−0.31 to −1.26), and

the combined data (−0.38 to −1.45), indicating an abundance of low-frequency alleles, or singleton mutations (Table 4). Similar trends are seen in F_u 's F_s (Table 4). When examining

Table 4 Demographic parameters for each population and for the entire populations of the spider-tailed viper in western Iran. Abbreviations: Comb., combined data; Seq. no., number of sequences used; h. no., number of haplotypes; rg, raggedness index; *, significance at $P < 0.05$; **, significance at $P < 0.01$; ***, significance at $P < 0.001$. The minimum number of sequences required to calculate Tajima's D and Fu's F_s is 4.

Population	Seq. no.	Gene	h. no.	Tajima's D	Fu's F_s	R_2	rg	Ka/Ks (P)
1	9	<i>Cytb</i>	5	-0.91	-1.59	0.15	0.12	0.000 (0.035)
	9	<i>CR1</i>	2	-1.08	-0.26	0.31	0.36	–
	9	Comb.	5	-1.07	-1.26	0.14	0.06	–
2	13	<i>Cytb</i>	9	-1.58	-3.53*	0.15	0.97	0.152 (0.048)
	14	<i>CR1</i>	14	-0.46	-3.19*	0.13	0.02	–
	13	Comb.	13	-0.64	-2.59	0.13	0.03	–
3	9	<i>Cytb</i>	5	-0.61	-0.61	0.13	0.08	0.000 (0.241)
	9	<i>CR1</i>	8	-0.31	-0.77	0.16	0.03	–
	9	Comb.	8	-0.38	-0.42	0.15	0.03	–
4	3	<i>Cytb</i>	3	–	–	0.31	0.44	0.107 (0.108)
	3	<i>CR1</i>	3	–	–	0.26	0.67	–
	3	Comb.	3	–	–	0.23	1.11	–
5	10	<i>Cytb</i>	5	-1.51	-0.025	0.12	0.009	0.214 (0.267)
	11	<i>CR1</i>	9	-1.26	-5.17**	0.09	0.05	–
	10	Comb.	10	-1.45	-6.01**	0.10	0.09	–
Populations combined	44	<i>Cytb</i>	25	0.09	-4.01**	0.12	0.009	0.074 (0.427)
	46	<i>CR1</i>	36	-0.70	-9.35***	0.09	0.003	–
	44	Comb.	39	-0.51	-10.16***	0.10	0.002	–

all populations combined, Tajima's D was positive only for *Cytb*, while Fu's F_s remained negative and significant ($P < 0.05$; Table 4). Small sample sizes may explain some non-significant results. Low values of rg and R_2 indices across all datasets indicate a high proportion of rare alleles within populations (Table 4), a pattern also evident in the haplotype networks with the widespread presence of singleton haplotypes (Figures 4–6).

Mismatch distribution analyses reveal that the combined data from populations 2–4 and all populations combined are not unimodal, deviating from models of sudden expansion (Figure 7), supported by non-significant values of Tajima's D and Fu's F_s . In contrast, populations 1 and 5 exhibit mismatch distributions consistent with sudden expansion (Figure 7). The raggedness index (rg) remains non-significant in overall and individual populations, supporting population stability (Table 4). We note that the small sample size in population 4 limits confidence in these analyses and will require a larger sample size to corroborate these findings.

When analyzing the separate and combined genes, Tajima's D remains negative and non-significant across individual populations and all populations combined, whereas Fu's F_s results are negative and significant in only some populations ($P < 0.05$; Table 4). Yet, Fu's F_s for all populations combined grouping is significantly negative ($P < 0.05$; Table 4). Demographic expansion estimates place the timing of

population growth around 7 million years ago ($\tau = 44.801$), with the total population increasing from an initial θ_0 of 8.480 to a θ_1 of 66.357.

The Ka/Ks ratio ranges from 0.00 to 0.214, with significant values only in populations 1 and 2 ($P < 0.05$), indicating negative selection on mitochondrial *Cytb* sequences (Table 4). The IBD test is non-significant within individual populations (i.e., intrapopulation; $P > 0.05$), but significant when considering the overall species' distribution ($r = 0.4536$; $P = 0.0001$). In binary comparisons, no significant correlation was observed between geographic distance and genetic differentiation among adjacent populations, whereas for more distant populations, this correlation was strongly significant. For example, populations 2 and 3 do not show genetic differentiation linked to geographic distance ($r = -0.057$; $P = 0.67$), whereas populations 1 and 5 exhibit a significant positive correlation ($r = 0.898$; $P = 0.0001$; Table 5).

4. Discussion

The results of genetic diversity and population genetic structure studies offer valuable insight for identifying distinct management units and play a key role in efforts to conserve the genetic health of species (Dutta *et al.*, 2020). This study was designed to analyze the genetic structure of *P. urarachnoides* in western Iran and to provide recommendations for preserving the genetic diversity of this species across its

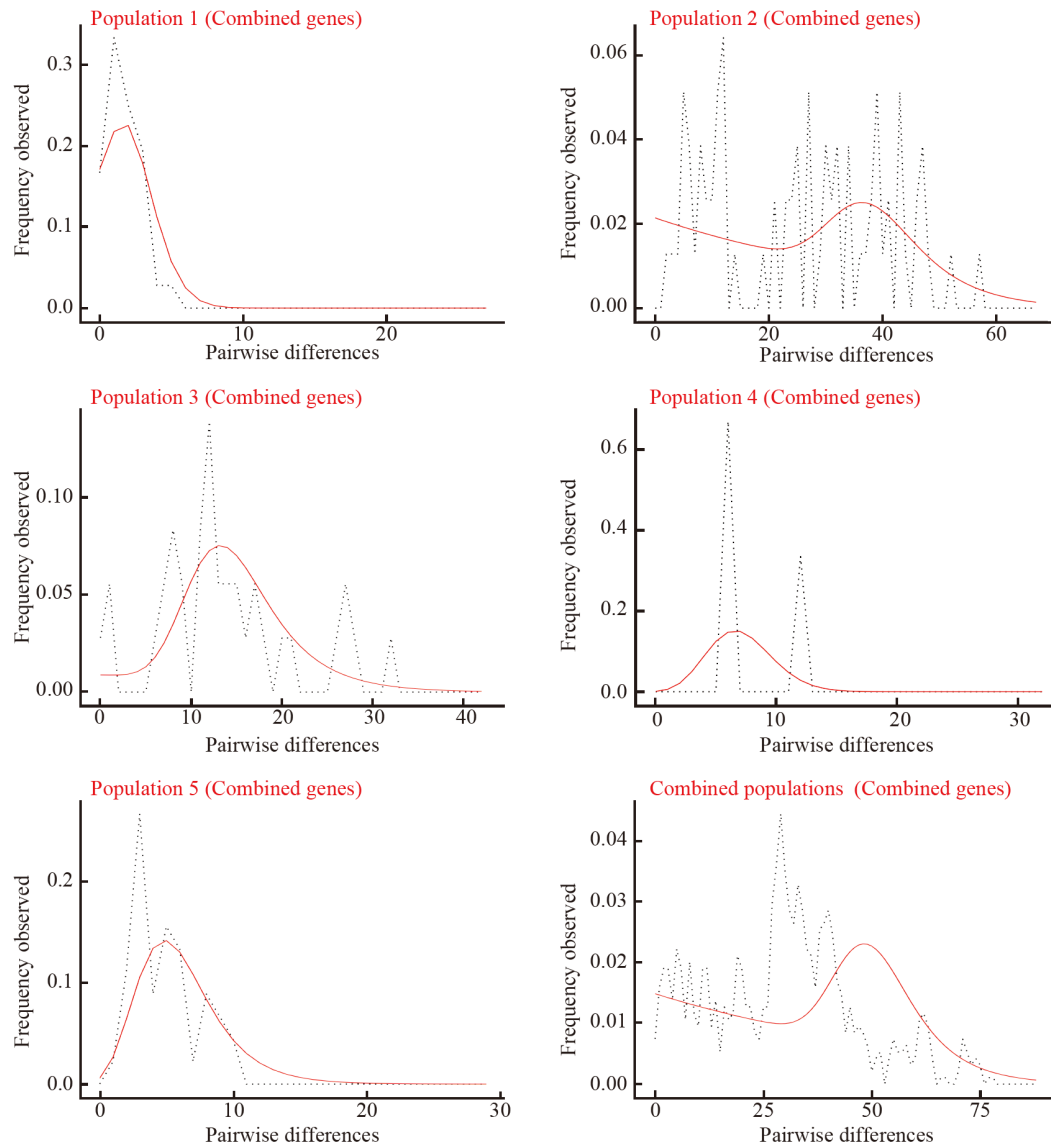


Figure 7 The mismatch distribution for the two genes (*Cytb* + *CRI*) indicates a lack of unimodal distribution among individual populations of the spider-tailed viper (except for population 1) and for the entire species in western Iran, based on pairwise sequence differences. The dotted line represents observed frequency, while the solid red line indicates expected frequency.

Table 5 Mantel test showing pairwise isolation by distance (IBD) among spider-tailed populations in western Iran. Values below and above the grey diagonal represent correlation (r) and significance (P), respectively. The symbols “**” and “***” indicate significance at $P < 0.01$ and $P < 0.001$, respectively.

Population	1	2	3	4	5
1		0.184	0.195	0.002**	0.0001***
2	0.124		0.674	0.0009***	0.0001***
3	0.087	-0.057		0.003**	0.0002***
4	0.712	0.625	0.778		0.008**
5	0.898	0.654	0.829	0.487	

range. Importantly, this is the first and most comprehensive study on the genetic structure and conservation genetics of this unique species.

The results of the study indicate that, at both the individual population and the overall population level, there are very high levels of Hd and, conversely, very low levels of π within the mitochondrial genome. This pattern (i.e., high Hd and low π) may indicate population expansion after a period of severe population decline, an event that provides the basis for the accumulation of new mutations (Avice *et al.*, 1984). Other factors that may influence this pattern include population isolation, inbreeding, or a combination of both. Low genetic diversity can negatively affect populations by increasing the risk of extinction (Parelli, 2013). Various studies have reported similar patterns across animal species, including corals (e.g., Moon Jellyfish *Aurelia coerulea* without an IUCN conservation assessment, Seo *et al.*, 2021), copepods (Gollner *et al.*, 2016), tapeworms (e.g., *Echinococcus granulosus* without an IUCN conservation assessment, Sharma *et al.*, 2013), fishes (e.g., *Gymnodictyus pachycheilus* with endangered status, Su *et al.*, 2014; and *Chitala chitala* with near threatened status, Dutta *et al.*, 2020), turtles (e.g., *Chelonia mydas* with endangered status, Bjørndal *et al.*, 2006), and mammals (e.g., *Bubalus bubalis* without an IUCN conservation assessment, Joshi *et al.*, 2013).

The haplotype networks for the spider-tailed viper in this study are not star-like. Star-like topologies usually indicate past genetic bottlenecks followed by recent population expansion (Slatkin and Hudson, 1991). Instead, the networks observed here show that each population has unique, population-specific haplotypes. This pattern, along with the absence of shared ancestral haplotypes, suggests a long history of isolation without recent demographic events, such as rapid expansions and limited gene flow among discrete populations. Instead, the haplotype network reflects stable, genetically distinct populations with separate evolutionary paths rather than recent demographic changes.

The *Cytb* haplotype network reveals five haplogroups, supporting the groupings suggested by SAMOVA and PCoA. In this network, common haplotypes were found between populations 2 and 3, but not among other groups, indicating isolation and limited gene exchange. The sharing of haplotypes (Hap_11 and Hap_12) between populations 2 and 3 hints at partial connectivity and gene flow between these populations.

Neutrality tests are sensitive indicators of population size changes, where significant negative values indicate population expansion (Delrieu-Trottin *et al.*, 2014). Demographic estimates show no signs of decline within individual populations or across the overall populations. The significantly negative Fu's F_s supports population expansion in western Iran. Low, non-significant raggedness index values

further suggest population stability. The negative and non-significant values of Tajima's D indicate an excess of singleton mutations. Dutta *et al.* (2020) noted that the founder effect may contribute to genetic constraints in populations, which may be contributing to the patterns observed within *P. urarachnoides*.

Pairwise F_{ST} values above 0.25 indicate profound genetic differentiation at the interpopulation level (Hartl and Clark, 1997). The F_{ST} results suggest varying levels of genetic differentiation, which are also supported by gene flow analyses. However, reliance on single-locus mitochondrial genes can sometimes lead to misinterpretation of gene flow, because mitochondrial DNA is non-recombining, maternally inherited (Gui *et al.*, 2020), and susceptible to bottlenecks (Wai *et al.*, 2008). Consequently, these factors may skew the interpretation of gene flow and population history. Therefore, to draw a comprehensive and accurate picture of population dynamics and gene flow, it is recommended to use both nuclear and mitochondrial markers. Future studies focused on this taxon using genome-scale data will be necessary to corroborate the findings presented here and can be used to explicitly test model-based hypotheses of demographic history (Myers *et al.*, 2020).

Genetic studies on various snake species have revealed diverse patterns of genetic variation. Some vipers, such as *Sistrurus catenatus* in North America and southern Canada (Gibbs *et al.*, 1997), *Vipera berus* in western Europe (Ursenbacher *et al.*, 2009), and *Vipera ursinii* in southern France (Ferchaud *et al.*, 2011), exhibit significant genetic structure similar to the findings presented here. Conversely, species such as the Australian *Hoplocephalus bungaroides* show low genetic differentiation (Dubey *et al.*, 2011). Similarly, *Coronella austriaca*, a small-territory colubrid, also exhibits low genetic structure (Vacher *et al.*, 2022). A recent study of the Massasauga Rattlesnake on Bois Blanc Island reported weak genetic structure and high gene flow, likely due to intact habitat conditions (Kudla *et al.*, 2021). Results from studies on this species in other geographic regions suggest that habitat quality and connectivity influence these patterns (Chiocchi and Gibbs, 2010; Sovic *et al.*, 2019). To fully understand the factors shaping genetic structure in the spider-tailed viper, further studies on the geology, historical climatology, and timing of population divergence are necessary.

A pattern of IBD among some populations in western Iran was present, indicating that geographic distance influences genetic differentiation. The observed population genetic structure likely results from limited gene flow and historical events (e.g., allopatric divergence with limited gene flow). Dispersal ability significantly affects patterns of IBD; for example, the smooth snake (*Coronella austriaca*) disperses over a range of about 6 km, more due to its low dispersal ability

than to habitat fragmentation, and significant IBD is observed at the regional, not local, level (Vacher *et al.*, 2022). Due to limited natural history data on the spider-tailed viper's home range, territory, philopatry, and dispersal capacity, pinpointing the main factors influencing the observed IBD is difficult. Nonetheless, ecological, behavioral, geographical, and environmental factors probably all play a role.

5. Conclusion

This research is the most detailed and comprehensive study of the genetic structure of the spider-tailed viper to date, providing valuable insights for the development of conservation programs for this species in western Iran. The findings of this research provide a valid basis for future IUCN assessments of the conservation status of this species. It is worth noting that *P. urarachnoides* is currently listed as Near Threatened (Mobaraki *et al.*, 2022), the observed population substructure should be taken into consideration in future assessments.

A detailed understanding of genetic structure is the foundation for developing effective management plans (Dutta *et al.*, 2020). Natural resource managers, especially those in the Iranian Department of Environment, need comprehensive knowledge about management units—particularly for endemic species under each IUCN category. This study identified five management units within the species' genetic structure, based on pairwise F_{ST} (ϕ_{ST}), gene flow (Nm), AMOVA, PCoA, and SAMOVA analyses. This foundational information will aid in devising conservation strategies for *P. urarachnoides*, which has ecological, conservation, and ecotourism significance. Given the strong population structure within this taxon, future studies should reassess the genetic structure across the entire distribution range of this species using genome-scale data. Additionally, including samples from outside Iran, especially from eastern Iraq, may significantly contribute to a more comprehensive understanding of population structure and the demographic history of *P. urarachnoides*. As a unique and endemic species that attracts increasing attention, conservation measures by the Iranian Department of Environment and policymakers will be necessary to preserve this viper and its associated ecosystem for future generations.

Acknowledgements We appreciate the support of the authorities of Yasouj University, Ilam University, and Iran DOE in conducting this research. We are also deeply indebted to the Iran National Science Foundation (INSF) for the financial support of this research (grant number 98011623). Our great thanks go to Prof Klaus Ulrich Joger (State

Museum of Natural History, Braunschweig, Germany) for his invaluable comments on this paper.

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Scientific Editor: Zhitong LYU

How to cite this article:

Fathinia B., Rashnavadi M., Rastegar-Pouyani N., Gowhari E., Myers E. 2026. Genetic structure of the spider-tailed viper, *Pseudocerastes urarachnoides* (Ophidia, Viperidae), in western Iran inferred from mitochondrial DNA sequences. *Asian Herpetol Res*, 17(2): 84–96. DOI: 10.3724/ahr.2095-0357.2025.0025. CSTR: 32242.14.ahr.2095-0357.2025.0025