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Molecular systematics of the genus *Pseudocerastes* (Ophidia: Viperidae) based on the mitochondrial cytochrome *b* gene

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Abstract: The false horned vipers of the genus *Pseudocerastes* consist of 3 species; all have been recorded in Iran. These include *Pseudocerastes persicus*, *P. fieldi*, and *P. urarachnoides*. Morphologically, the taxonomic border between *P. fieldi* and *P. persicus* is not as clear as that between *P. urarachnoides* and *P. persicus* or *P. fieldi*. Regarding the weak diagnostic characters differentiating *P. fieldi* from *P. persicus* and very robust characters separating *P. urarachnoides* from both, there may arise some uncertainty in the exact taxonomic status of *P. urarachnoides* and whether it should remain at the current specific level or be elevated to a distinct genus. Based on our sequence data from the mitochondrial cytochrome *b* gene, the taxonomic status of the 3 false horned vipers is confirmed. Although *P. fieldi* has equal genetic distance from *P. persicus* and *P. urarachnoides*, the spider-tailed viper is more closely related to *P. persicus* than *P. fieldi*. There are also some subdivisions among the populations of *P. persicus* in Iran.

Key words: Cytochrome *b*, genetic divergence, molecular systematics, *Pseudocerastes*, Viperidae, Iran

1. Introduction

The genus *Pseudocerastes* Boulenger, 1896 encompasses 3 false horned viper species, including *Pseudocerastes persicus* (Duméril, Bibron & Duméril, 1854); *Pseudocerastes fieldi* Schmidt, 1930; and *P. urarachnoides* Bostanchi, Anderson, Kami & Papenfuss, 2006 (Bostanchi et al., 2006; Fathinia et al., 2009; Fathinia and Rastegar-Pouyani, 2010). The genus has a wide and mostly Middle Eastern distribution in Egypt, Israel, Jordan, the extreme north of Saudi Arabia, Syria, Iraq, Iran, Pakistan, Afghanistan, and some outlying populations in northern Oman and the Musandam Peninsula (Leviton et al., 1992; Latifi, 2000; Bostanchi et al., 2006; Phelps, 2010).

Pseudocerastes urarachnoides is a remarkable taxon with recently known limited and narrow distribution in western regions of Kermanshah, Ilam, and the northern Khuzestan provinces in Iran. This viper has some fascinating characters such as highly rugose pholidosis and an arachnid-like caudal structure for attracting and luring prey (Fathinia et al., 2009). Although the morphological characters are strong enough to separate *P. urarachnoides* from the 2 other species, the exact taxonomic position of the spider-tailed viper using molecular markers has previously not been uncovered. In the current study, the exact taxonomic status of the Iranian spider-tailed viper is presented using partial sequences of the mitochondrial cytochrome *b* gene.

2. Materials and methods

2.1. Taxon sampling

The collection localities of all specimens used in this study are given in Table 1 together with the data retrieved from GenBank. Tissue samples taken from individuals of each of the following species, collected from 6 separate sites in Iran (Figure 1), were used for molecular studies: 9 specimens of *P. persicus* (4 from Ilam Province; 2 from Hormozgan Province; 1 from Sistan and Baluchistan Province; 1 from Damghan, Semnan Province; and 1 from Maybod, Yazd Province) and 3 specimens of *P. urarachnoides* (all from Ilam Province).

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Table 1. Information of the specimens used in the study.

Taxon	Field number	Accession number	Locality
Eristicophis macmahonii	------	HM1794641	Pakistan
Eristicophis macmahonii	------	AJ2757111	Pakistan
Pseudocerastes fieldi	------	AJ2757161	Israel
Pseudocerastes persicus	20	KF314711	Bazman, Sistan and Baluchistan Province, Iran
Pseudocerastes persicus	21	KF314712	Bandar-e-Abbas, Hormozgan Province, Iran
Pseudocerastes persicus	22	KF314713	Saraj, Hormozgan Province, Iran
Pseudocerastes persicus	25	KF314705	Damghan, Semnan Province, Iran
Pseudocerastes persicus	27	KF314706	Maybod, Yazd Province, Iran
Pseudocerastes persicus	30	KF314707	Ilam, Ilam Province, Iran
Pseudocerastes persicus	30k	KF314708	Ilam, Ilam Province, Iran
Pseudocerastes persicus	32	KF314709	Ilam, Ilam Province, Iran
Pseudocerastes persicus	1000	KF314710	Ilam, Ilam Province, Iran
Pseudocerastes urarachnoides	24	KF314714	Ilam, Ilam Province, Iran
Pseudocerastes urarachnoides	31k	KF314715	Ilam, Ilam Province, Iran
Pseudocerastes urarachnoides	1064	KF314716	Ilam, Ilam Province, Iran

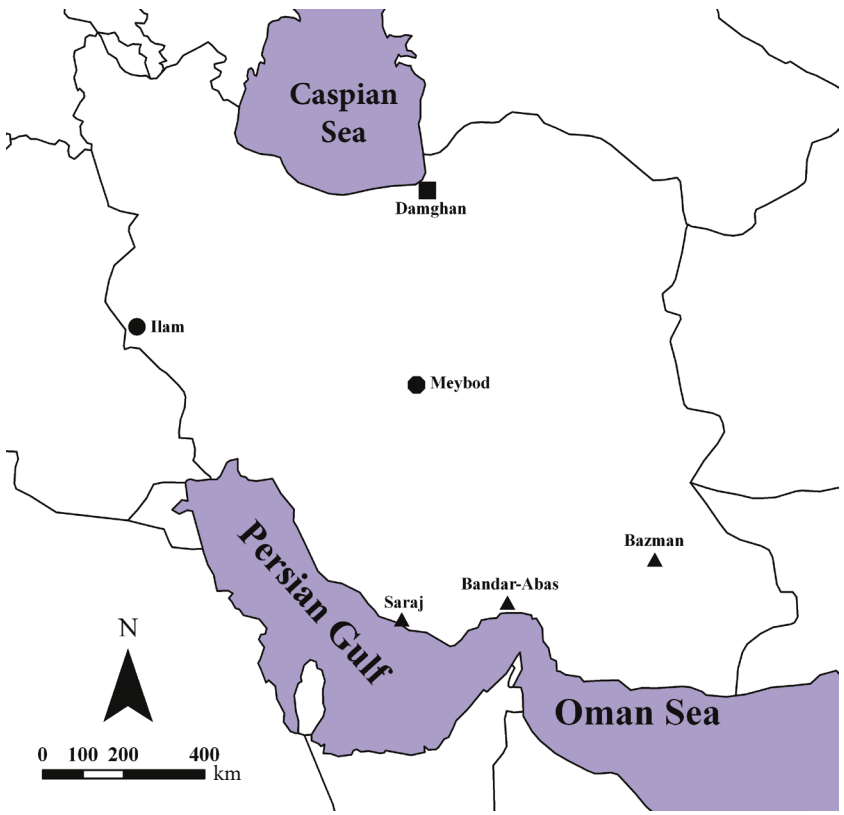

Figure 1. Map showing the localities of collected Iranian specimens used in the study. Solid circle for the western specimens of *P. urarachnoides* (KF314714–16) and *P. persicus* (KF314707–10); solid square for the only specimen from the northern populations of *P. persicus* (KF314705); solid octagon for the only specimen of the central populations of *P. persicus* (KF314706); and solid triangle for specimens of the southern populations (KF314711–13).

2.2. DNA extraction, polymerase chain reaction, and sequencing

Total genomic DNA was extracted from blood and muscle samples preserved in absolute ethanol. The standard method of Sambrook et al. (1989) was followed for extracting genomic DNA. Polymerase chain reaction (PCR) was conducted in 0.2-mL reactions with 1–2 µL of extracted DNA, 2.5 µL of the PCR buffer, 2 µL of 2.5 mM dNTPs, and 0.2 µL of Taq DNA polymerase in each case. For amplifying cytochrome *b*, 1 µL of forward primer (Cerastes-100f, 5'-CAATACTATTAGCCTGCCTAATAA-3') and 1 µL of reverse primer (Cerastes-700r, 5'-GGYGTCCTTGAAGGAGTGGTATGG-3') were used in each reaction. The PCRs were conducted at the following temperatures: an initial denaturation at 95 °C for 3 min; 38 cycles of denaturation at 95 °C for 40 s, annealing at 50 °C for 40 s, and extension at 72 °C for 70 s; and a final elongation at 72 °C for 10 min. The reaction was stopped at 4 °C within 10 min. The 538-bp fragment was sequenced in both directions using the same primers.

2.3. Phylogenetic analyses

The sequences were aligned using Clustal W as implemented in the BioEdit Sequence Alignment Editor 7.0.5.3 (Hall, 1999), with later corrections by eye. To exclude possible numts (nuclear mitochondrial sequences), the cytochrome *b* nucleotide sequences were translated to amino acid sequences for determining the presence of any stop codons or nonsense mutations using MEGA 6.05 (Tamura et al., 2013). Each of the cytochrome *b* sequences was checked by DnaSP v. 5.10 (Librado and Rozas, 2009) to determine unique haplotypes.

Two methods of phylogenetic reconstruction, maximum parsimony (MP) using PAUP 4.0b10 (Swofford, 2001) and neighbor-joining (NJ; Saitou and Nei, 1987), as implemented in the program MEGA 6.05 (Tamura et al., 2013), were used. MP analyses were conducted with heuristic searches, tree bisection-reconnection, and simple stepwise addition. In performing MP analyses we used equal weighting for all characters as well as the presence of codon third positions. In choosing to emphasize equal weighting for all nucleotide sites, we make the assumption that at the taxonomic level of our ingroup, the value of increased resolution at subterminal nodes provided by codon third positions will not outweigh possible perturbations at deeper nodes. NJ analysis was done in MEGA 6.05 with 6000 replications using the K2P model and a gamma parameter of 1 for rates among sites.

To assess nodal supports in MP topology, bootstrap analysis was conducted in PAUP 4.0b10 with the heuristic search approach with 8000 replications using 100 random repetitions for each replication (Felsenstein, 1985). Different levels of bootstrapping support were defined as weak (50%–69%), moderate (70%–89%), and strong (90%–100%). Because *Eristicophis* is the closest relative to *Pseudocerastes* (Wüster et al., 2008; Stümpel and Joger, 2009), 2 cytochrome *b* sequences of *Eristicophis macmahonii* from Pakistan were retrieved from GenBank and included in the analyses for rooting the reconstructed trees.

3. Results

The cytochrome *b* dataset comprised 538 aligned sites, of which 407 were constant, while 38 variable characters were parsimony-uninformative and 93 were parsimony-informative. The base frequency distribution was as follows: T = 26%, C = 34.6%, A = 26%, and G = 13.5%. Results of the analysis by DnaSP v. 5.10 showed no presence of redundant haplotypes in the dataset (Table 2). MP analysis yielded 2 equally parsimonious cladograms with 204 evolutionary steps with the following characteristics: consistency index (CI) = 0.7892, homoplasy index (HI) = 0.2108, and retention index (RI) = 0.8238. The tree topology and branching pattern in 2 equally parsimonious trees were slightly different only in the position of 1 of the terminal nodes, i.e. *P. persicus* Raja 20. Figure 2 shows the strict consensus tree of these 2 equally parsimonious trees as calculated with PAUP 4.0b10. The tree topology produced by NJ analysis was the same as that of MP regarding the general topology.

As seen from the reconstructed tree, the first dichotomy separates *P. fieldi* from its sister taxa, *P. persicus* and *P. urarachnoides*, with a strong bootstrap value of 100%, in both MP and NJ, indicating monophyly of all 3 species of *Pseudocerastes*. The second dichotomy separates *P. urarachnoides* from *P. persicus* with moderate bootstrap values of 69% in MP and 72% in NJ, indicating monophyly of the 2 taxa. Thus, the recovered phylogeny presented here suggests that the Iranian spider-tailed viper (*P. urarachnoides*) is the sister taxon of the Persian false horned viper (*P. persicus*).

Intraspecific subdivision of the Iranian populations of *P. persicus* is also demonstrated in the trees. The most basal dichotomy in the *persicus* clade separates Maybod-Pakistan-Damghan (M-P-D) specimens from the sister clade composed of the remaining western and southern (W-S) populations. M-P-D specimens are a sister taxon to W-S populations, hence forming a monophyletic clade with strong bootstrap values of 98% in MP and 99% in NJ. However, there is ambiguity in the exact taxonomic status of the M-P-D populations only in MP. The second divergence in the *persicus* clade separates the western population from the southern one. The 2 populations form a monophyletic clade with strong bootstrap values of 91% in MP and 96% in NJ. However, the southern population shows uncertainty in its taxonomic position in MP topology.

Table 2. Variability in nucleotide positions and diversity among partial sequences of cytochrome *b* (538 bp) for 14 haplotypes used in the study.

Haplotypes	Polymorphic sites Singleton variable sites (2 variants): 34 40 63 75 87 99 101 103 132 174 180 183 205 220 222 223 224 225 231 252 294 307 325 334 337 339 345 360 366 412 438 441 451 476 504 516 519 528 537 Parsimony informative sites (2 variants): 55 58 69 78 97 108 117 123 126 135 141 162 163 171 216 234 240 243 249 258 264 291 297 315 321 327 348 349 351 354 378 387 405 414 420 433 484 486 489 507 536 Singleton variable sites (3 variants): 456 471 Parsimony informative sites (3 variants): 36 124 201 255 267 276 336 357 417 421 459 492 498 525	Taxon	Frequency
1	ATTAGTCCACCAATCCCCGCATACTGCAAGTCACTCGACTTCAAGCATATCTCCACCTGAACAAAGCCCCTAGCACAACTCCACAGGACCACGCAC	*P.fieldi*.AJ275716	1
2	..C..CTTCT.....T..G.CC.GT.CTCACTAGTCCTGCAAC.TCTCT.GTGCACGTG.GCT.T...TTGTCGAATGG.G.CCT.G.C...	*P.persicus*.AJ27571	1
3	G.C..CT.CT.....A.T...TCCT.T.CTCAC.A.TCCTG.AGC.T.TCT.GTGCACGT..TCT.T...TTGTC.AC.GG.G.CC....A.T.	*P.persicus*Raja20	1
4	..C..C..CT.....T.T.T...TCC..T.CTCAC.A.TCCTGCAGC.T.TCT.GTGCACGT.GTCT.T...TTGTC.AC.GG.G.CC....A.TA	*P.persicus*Raja21	1
5	..C..CT.CT.....T.T.T..GTCC..T.CTCAC.A.TCCTGCAGC.T.TCT.GTGCACGT.GTCT.T...TTGTC.AC.GG.G.CC....A.T.	*P.persicus*Raja22	1
6	..C..CT.CT.....T...T.C..T.CTCAC.AGTCCTGCAGC.TCTCT..TGCACGT..GCT...C.TT.TC.ACTGG.G.CC....A...	*P.persicus*25	1
7	..C..CT.CT.....T..GTCC..T.CTCAC.AGTCCTGCAAC.T.T.TTGTGCAC.TG.GCT.T...T.GTC.ACTAG.G.CT...TCT..	*P.persicus*27	1
8	..CG.CT.CTG.....A.T...TCC..T.CTCAC.A.TCCTGCAGC.T.TCT.GTGCACGT.GTCT.T...TTGTC.AC.GG.G.CC....A...	*P.persicus*30	1
9	..CG.CT.CT...A...A.T...TCC..TACTCAC.A.TCCTGCAGC.T.TCT.GTGCACGT.GTCT.T...TTGTC.AC.GG.G.CC....A...	*P.persicus*30K	1
10	.ACG.CT.CTG.....A.T...TCC..T.CTCAC.A.TCCTGCAGC.T.TCT.GTGCACGT.GTCT.T...TTGTC.AC.GG.G.CC....A...	*P.persicus*32	1
11	..CG.CT.CTGCC....A.T...TCC..T.CTCAC.A.TCCTGCAGC.T.TCT.GTGCACGT.GTCT.T...TTGTC.AC.GG.G.CC....A...	*P.persicus*1000	1
12	.CC.AC...T....T.A.ATGC..CC..C..TCAC.AG...TCC.AGCTCT....TTCAC...GGCTATT.GCTT.C.ACGGGT.ATT.T..A...	*P.urarachnoides*24	1
13	.CC.AC...T....T.A.ATGC..CC..C..TCAC.AG..CTCC.AGCTCT....TTCAC....GCTATT.GCTT.C.ACGGGT.ATT.T..A...	*P.urarachnoides*31K	1
14	.CC.AC...T....T.A.ATGC..CC..C..TCAC.AG...TCCAAGCTCT....TTCAC...GGCTATT.GCTT.C.ACGGGT.ATT.T..A...	*P.urarachnoides*1064	1

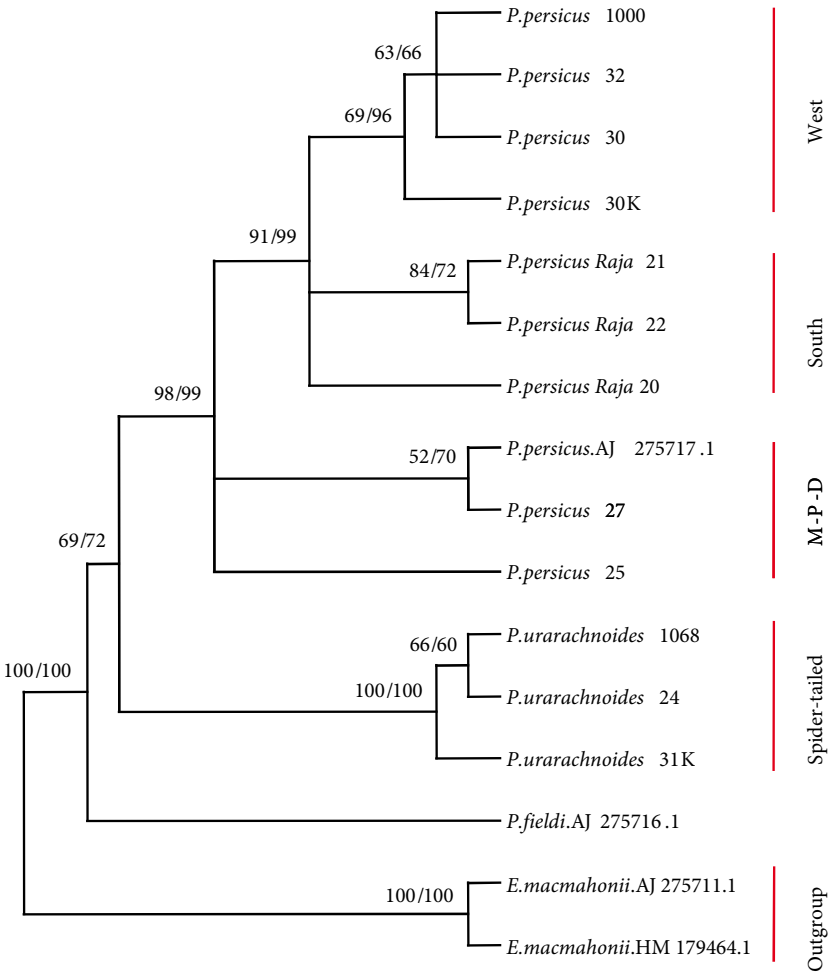

Figure 2. Maximum parsimony strict consensus tree of the 2 most parsimonious trees recovered in the analysis. *Eristicophis macmahonii* was used as an outgroup taxon for rooting the tree. The phylogenetic tree produced by NJ has the same topology as the presented tree with regard to the major lineages. Numbers above branches on the right side of slashes are bootstrap percentages (8000 replicates) for MP and on the left side are bootstrap percentages (6000 replicates) for NJ.

The Kimura 2-parameter method, implemented in MEGA 6.05, was employed in calculating the genetic distances. Genetic difference in partial cytochrome *b* sequence is 19% between *E. macmahonii* and *P. fieldi*, 17% between *E. macmahonii* and *P. persicus*, and 18% between *E. macmahonii* and *P. urarachnoides*. *P. fieldi* shows the same genetic difference of 14% with both *P. persicus* and *P. urarachnoides*. Divergence in cytochrome *b* sequence between *P. urarachnoides* and *P. persicus* is around 10%, whereas that among different populations of *P. persicus* varies from 2% to 4% (see Table 3 for more details).

4. Discussion

This is the first attempt to uncover the molecular systematics of the genus *Pseudocerastes*. *Pseudocerastes urarachnoides* has unique morphological characters that strongly separate it from *P. persicus* and *P. fieldi*. These characters are as follows: fewer subcaudal plates (15 pairs at most versus 52 pairs in *P. persicus*), rugose pholidosis, and the arachnid-like structure of the tail tip, which gives it the common name 'spider-tailed viper' (Bostanchi et al., 2006; Fathinia and Rastegar-Pouyani, 2010). These 3 characters are unique features in this viper, absent in its congeners; hence, they should be regarded as autapomorphies.

Unlike the weak and overlapping morphological characters that separate *P. fieldi* from *P. persicus* (Bostanchi et al., 2006; Fathinia and Rastegar-Pouyani, 2010; Phelps, 2010), the morphologically diagnostic characters separating *P. urarachnoides* from both *P. fieldi* and *P. persicus* are quite robust, and one may be uncertain whether, based on simple morphological grounds, to consider it as a species within *Pseudocerastes* or a distinct

Table 3. Evolutionary genetic divergence over sequence pairs among major clades calculated by K2-p model.

	E. macmahonii	*P. fieldi*	*P. urarachnoides*	*P. persicus*_West	*P. persicus*_P-M-D
E. macmahonii					
P. fieldi	19%				
P. urarachnoides	18%	14%			
*P. persicus*_West	16%	14%	10%		
*P. persicus*_P-M-D	17%	14%	10%	4%	
*P. persicus*_South	17%	14%	11%	2%	4%

genus. According to the current molecular survey, the spider-tailed viper falls within the genus *Pseudocerastes* but is well separated from *P. fieldi*, with around 14% DNA divergence, and from *P. persicus*, with around 10% DNA divergence (Table 3). Hence, the taxonomic status of *P. urarachnoides* as a distinct species belonging to *Pseudocerastes* is confirmed in the present study. Table 3 also indicates that *P. fieldi* has equal genetic distance to both *P. persicus* or *P. urarachnoides*.

P. persicus has a wide distribution range in Iran, Pakistan, Afghanistan, and Oman (Bostanchi et al., 2006). Therefore, the presence of some degree of morphological, ecological, molecular, and even behavioral divergences among different geographically distant populations of this species is understandable. Only 9 specimens of *P. persicus* representing the populations of West, South, Central, and North Iran, as well as 1 sequence of *P. persicus* from Pakistan, were used in the analysis. The results showed significant separation of different populations, with around 2% to 4% of mtDNA divergence (Table 3). Due to inadequate sampling, the exact taxonomic statuses of the central and northern populations of Iran as well as Pakistani populations have not been resolved (only 1 specimen of each population was employed in the analysis). Hence, more specimens and genes must be included in future analyses to determine taxonomic status and to reveal geographic variations among different populations of *P. persicus*. Moreover, some ambiguity is seen within the southern population, but this unresolved pattern is most likely caused by inadequate sampling.

The robustness of the taxonomic status of the 3 false horned vipers is confirmed at the species level in this study, as well as the conspicuous finding that *P. urarachnoides* is nested within this genus. As this study was based on only one mitochondrial gene and a limited number of specimens were included, future work should include more robust sampling, sequencing of additional genes, and a detailed examination of morphological variation.

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