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Original Article

DNA barcoding of *Aphanius vladykovi* from different habitats in Chaharmahal va Bakhtiari Province, Iran

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Abstract: This study was aimed to reveal the possible cryptic diversity of the *Aphanius vladykovi* populations in the Chaharmahal va Bakhtiari Province, Iran using mitochondrial cytochrome-c oxidase subunit I (COI). A total of 30 specimens from the Beheshtabad River, Choghakhor and Gandoman Wetlands, and Brovi, Shalamzar, and Balagholi springs from the Chaharmahal va Bakhtiari Province were collected. The maximum within-population genetic distance based on K2P was 0.28% and this distance was 0.22% between populations of Gandoman and Brovi with Shalmazar, whereas the least genetic distance was observed between Choghakhor and Beheshtabad (0.09%). A total of six haplotypes were observed between the studied specimens. Maximum Likelihood (ML) and Neighbor-Joining (NJ) trees reconstructed and all haplotypes from *A. vladykovi* specimens collected from non-type localities nested in one group with *A. vladykovi* from Choghakhor wetland i.e. type locality. The results of this study detected no cryptic diversity in *A. vladykovi* inhabiting different habitats in the studied region. Hence, it is proposed to consider all the studied populations in conservation measures related to *A. vladycovi*.

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Introduction

The family Aphaniidae is composed of the genus *Aphanius* with 15 reported species from Iranian inland waters (Esmaeili et al., 2018). The greatest diversity of this genus is found in the Near East, particularly Iran and Turkey (Teimori et al., 2018; Cicek at al., 2018). The members of this genus can adapt to a wide range of environmental parameters (Cavraro et al., 2017), inhabiting rivers, wetlands, pools, springs, and Qanats in semiarid and arid regions. Therefore, this ability makes it possible for them to form different isolated populations causing diversification and radiation of different taxa. Hence, there may also be more unknown diversity in this genus that should be explored using molecular tools.

Aphanius vladykovi Coad, 1988, described from Choghakhor Wetland (31°57'N, 51°01'E) in Zagros Mountains of Iran (Coad, 2017), is found in the Karoun river drainage in the Chaharmahal va Bakhtiari Province, Iran (Coad, 2017; Esmaeili et al., 2018). Male *A. vladykovi* is characterized by a light transverse strip on the body, while female has dark spots on both sides of the body (Coad, 2017). The populations of this species inhabit different isolated habitats in the Chaharmahal va Bakhtiari Province. In previous studies, *A. vladykovi* from its type locality has been compared to different *Aphanius* spp. (Esmaeili et al., 2014; Teimoori et al., 2012), but there is no molecular data of different populations of this species.

Mitochondrial DNA (mtDNA) that widely used for identifying cryptic fish species (Rezvani Gilkole, 1997; Ivanova et al., 2007; Asgharian et al., 2011), play an important role in taxonomic studies and phylogenetic inferences (Dabert, 2006; Yang et al., 2010). During the past decade a movement known as DNA barcoding has started in which scientists from all over the world produce partial sequences of a standard gene region of around 650 base pairs using universal primers to identify different animal

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Table 1. Details of the sampling stations.

Locality	Sampling date	Coordinates	
		Lon	Lat
Brovi Spring	2017-01-02	32°16'48.3"N	50°59'25.1"E
Balagholi Spring	2017-06-03	32°05'02.8"N	50°42'47.2"E
Behesht-Abad River	2017-01-03	32°05'09.3"N	50°39'41.9"E
Choghkhor Wetland	2016-11-28	31°56'05.1"N	50°54'29.7"E
Gandoman Wetland	2017-06-03	31°49'40.2"N	51°06'01.5"E
Shalamzar Spring	2016-11-28	32°01'27.4"N	50°49'18.8"E

species (Kerr et al., 2007). The purpose of DNA barcoding is to improve the identification of species and to discover new species by studying patterns of sequence differentiation in a standard region in the genome (http://www.boldsystems.org/). The partial COI sequence is used to study different groups, especially at species and population levels (Tala et al., 2011; Asgharian et al., 2011; Hashemzadeh Segherloo et al., 2012b). DNA barcodes have been used successfully for delimitation of species in more than 90% of animal species studied (Ward et al., 2005; Hajibabaei et al., 2006; Hubert et al., 2008). In this study, the *A. vladykovi* populations in the Chaharmahal va Bakhtiari Province, Iran was investigated in different localities to explore possible cryptic diversity.

Materials and Methods

Fish were collected using a scope net from different localities, including Beheshtabad River, Choghakhor and Gandoman Wetlands, and Shalamzar, Berovi and Balaghololi springs (Table 1). The right pectoral-fin of five specimens from each locality were clipped and subsequently fixed in 96% ethanol. DNA extraction was performed using salt extraction method (Aljanabi and Martinez, 1997). For amplification of the COI gene, the primers *FishF1*-5′TCAACCAACCACAAA GACATTGGCAC3′ and *FishR1*- 5′TAGACTTCTG GGTGGCCAAAGAATCA3′ were used (Hubert et al., 2008). The PCR reaction (25 µl per reaction) contained 2.5 µl of 10× buffer, 0.5 µl of (50 mM) $MgCl_2$, 0.5 µl of (25 mM) deoxynucleotide triphosphate (dNTP), 0.5 µl (10 µm) of each primer, 0.5 µl of Taq polymerase (5 U μl^{-1}), 2 µl of total DNA, and 18 µl of H_2O. Amplification cycles were: denaturation for 2 min at 94°C; 30 cycles at 94°C for 1 min, 59°C for 0.5 min, 72°C for 0.5 min, and a final extension for 2 min at 72°C. The 5' end of COI with an approximate length of 652 nucleotides was amplified. The 5' end of the COI gene was sequenced on an ABI-3130xl sequencer using forward primer. The sequences were checked and edited visually based on chromatograms, compared to sequences in each population, and GenBank.

The sequences were translated to their respective amino acid sequences with MEGA7 (Kumar et al., 2016) to see whether the confirmed mutation had led to any change in protein sequences compared to standard amino acid sequence of COI or not. The sequences were compared to the published *Aphanius* sequences using BLAST search in GenBank (Altschul et al., 1997) to find similar sequences (Table 2). A common 584 bp length of the selected COI segment was used for further analyses. The gene diversity indices, including number of polymorphic sites, number of haplotypes (h), haplotype diversity (Hd), and nucleotide diversity (Pi) were calculated with DnaSP v6 (Rozas et al., 2017). To provide a quantitative measure of the sequence divergence, the divergence among studied populations, genetic distances between sequences were calculated based on the Kimura two-parameter (K2P) model of sequence divergence with MEGA7. Phylogenic trees were reconstructed based on maximum likelihood (ML) and neighbor joining (NJ) methods with MEGA7 and RaxML (Silvestro and Michalak, 2012). The best fit model of sequence evolution to be used in Maximum likelihood approach was selected with the modeltest

Table 2. List of sequences used from NCBI-GenBank.

Species	Accession	Distribution
Aphanius alexandri	KJ552647	Middle East.
Aphanius almiriensis	KJ552360	Europe: Greece.
Aphanius anatoliae	KJ552467	Asia: endemic to Turkey.
Aphanius asquamatus	KJ834543	Asia: Lake Hazar, eastern Anatolia and Iran.
Aphanius baeticus	KJ552418	Europe: Spain along Atlantic coast between Huelva and Gulf of Cadiz.
Aphanius danfordii	KJ834531	Asia: Eastern Turkey.
Aphanius fasciatus	KJ552516	Europe: France, Italy, Slovenia, Croatia, Albania, Greece and Montenegro. Asia: Turkey. Mediterranean basin: North Africa from Egypt to eastern Algeria.
Aphanius fontinalis	KJ552742	Asia: endemic to Turkey.
Aphanius fontinalis	KJ552560.	
Aphanius iconii	KJ552688	Asia: endemic to Turkey.
Aphanius iconii	KJ552481	
Aphanius maeandricus	KJ552515	Asia: endemic to Turkey.
Aphanius mento	KJ552511	Middle East.
Aphanius mentoides	KJ552397	Middle East.
Aphanius orontis	KJ552683	Middle East.
Aphanius saldae	KJ552398	Asia: Central Turkey.
Aphanius saourensis	KJ552623	Africa: Algeria.
Aphanius similis	KJ552500	Middle East.
Aphanius similis	KJ552367	Middle East.
Aphanius sureyanus	KJ834526	Asia: Central Turkey.
Aphanius transgrediens	KJ552368	Asia: Central Turkey.
Bathygobius sp.	KT357897	Southwest Pacific: Australia, including the Lord Howe and Norfolk islands.
Canthigaster rivulata	JF952693	Indo-Pacific: East Africa south to Natal, South Africa and east to Hawaii, north to southern Japan, south to northwestern Australia.
Canthigaster smithae	JF493017	Western Indian Ocean: Agalega Islands, Mauritius to Durban, South Africa. Also Maldives.
Parupeneus spilurus	DQ107798	Western Pacific: Japan to Western Australia, New Caledonia and northern New Zealand. Recently reported from Tonga.
Pseudamia gelatinosa	FJ346820	Indo-Pacific: Red Sea and East Africa to French Polynesia, north to Ryukyu Islands, south to Sydney Harbor, New South Wales (Australia).

Table 3. K2P sequence divergence of the studied populations.

Group	Within Group	Between Group				
Shalamzar (1)	0.28					
Gandoman (2)	0.2	0.22				
Choghakhor (3)	0.17	0.21	0.17			
Brovi (4)	0.08	0.22	0.17	0.21		
Beheshtabad (5)	0	0.17	0.13	0.09	0.21	
Balagholi (6)	0	0.17	0.13	0.17	0.14	0.17

utility implemented in MEGA7. To check for the robustness of each branch on phylogenetic trees a bootstrap test with 1000 replicates was used for both NJ and ML approaches. To visualize the mutational relationships among the haplotypes identified in this study and the haplotypes retrieved from GenBank a rooted TCS network was reconstructed with PopART-1.7 (http://popart.otago.ac.nz). To root the phylogenetic tree *Bathygobius* sp., *Canthigaster rivulata*, *Canthigaster smithae*, *Parupeneus spilurus*, and *Pseudamia gelatinosa* were used as out-group species (Table 2).

Results

In this study six haplotypes were resolved, which differ from one another by 1-3 mutations (Fig. 1). Haplotype diversity (Hd) and nucleotide diversity (Pi) were 0.680 and 0.001, respectively. The haplotypes *A. vla*2 was the most frequent one observed in all populations except Beheshtabad River. The second

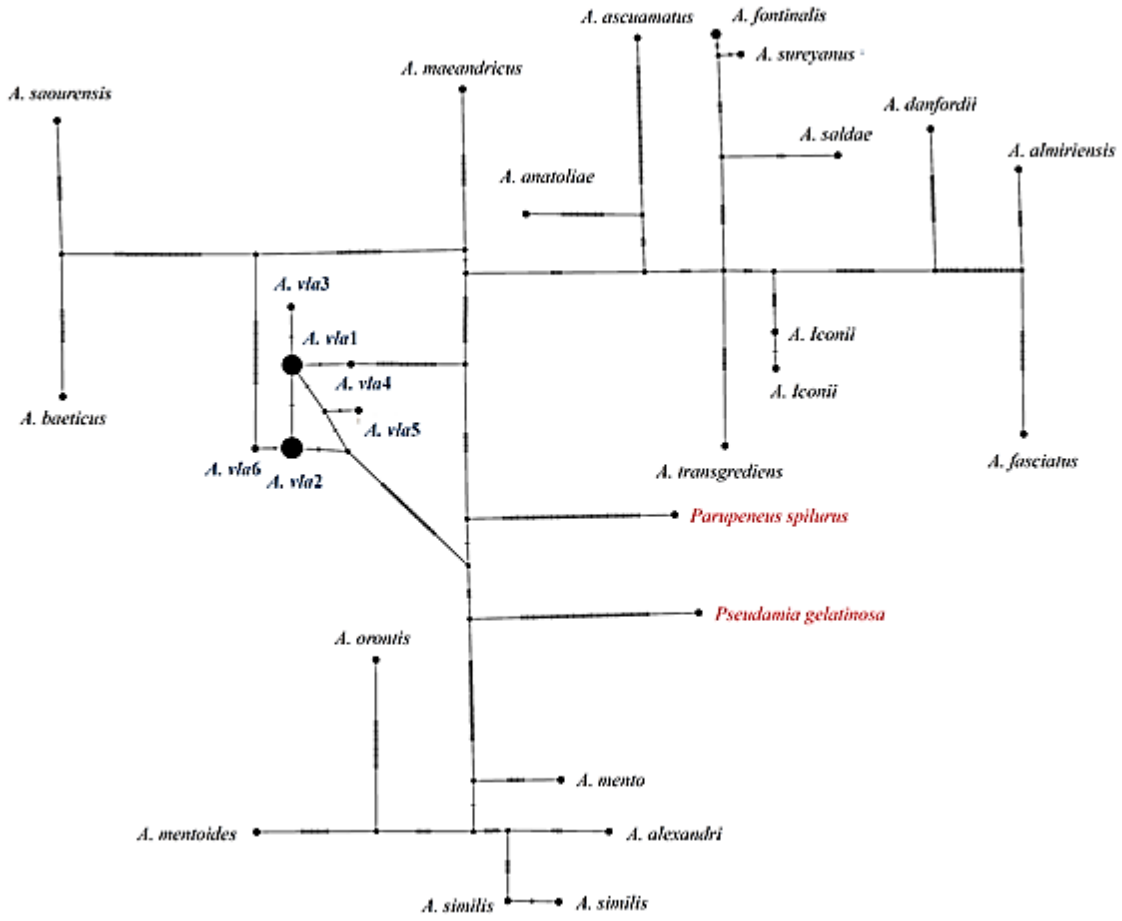

Figure 1. The COI haplotype network showing mutational relationship of sequences produced in this study and sequences from GenBank. The hatch lines along the connection lines denote the number of mutational steps between each pair of haplotypes. Black circles are probable haplotypes from which different mutational paths radiate. Abbreviations: A. *vla: A. vladykovi.*

frequent haplotype was *A. vla*1 observed in all populations except in Balagholi and Berovi springs. Other haplotypes were population specific.

The maximum within group K2P sequence differentiation was calculated in the Shalamzar population (0.28%). The maximum between group genetic distance was calculated between the Gondoman and Berovi populations and Shalamazar population (0.22%) and the minimum genetic distance was found between the Choghakhor and Beheshtabad populations (0.9%; Table 3).

The maximum likelihood and neighbor-joining phylogenic trees were similar in topology (Fig. 2). *Aphanius* spp. used for reconstruction of phylograms were nested in three monophyletic groups including *A. vladykovi* (BS= 100), *A. orontis, A.mento, A. mentoides, A. alexondri,* and *A. similis* (Middle Eastern group; group II; BS=100), and *A. baeticus, A. saourensis*, *A. almiriensis, A. facsiatus, A. asquamatus, A. danfordii, A. anatoliae, A. maeandricus, A. iconii, A. transgrediens, A. saldae, A. fontinalis,* and *A. sureyanus* (European-Anatolian group; group III; BS=66-67). All *A. vladykovi* specimens collected from different localities, nested in a monophyletic group with absolute bootstrap support on both NJ and ML phylograms (BS=100). *Aphanius* spp. from Europe, North Africa, and the Middle East nested in the third group (III).

Discussions

The intra-species sequence differentiation in freshwater fishes had been reported to be 0.27% and the intra-genus divergence among different freshwater fishes falls in a range of 0-19.3%, with an average of 8.37% (Hubert et al., 2008). The divergence values observed between the studied populations of *A.*

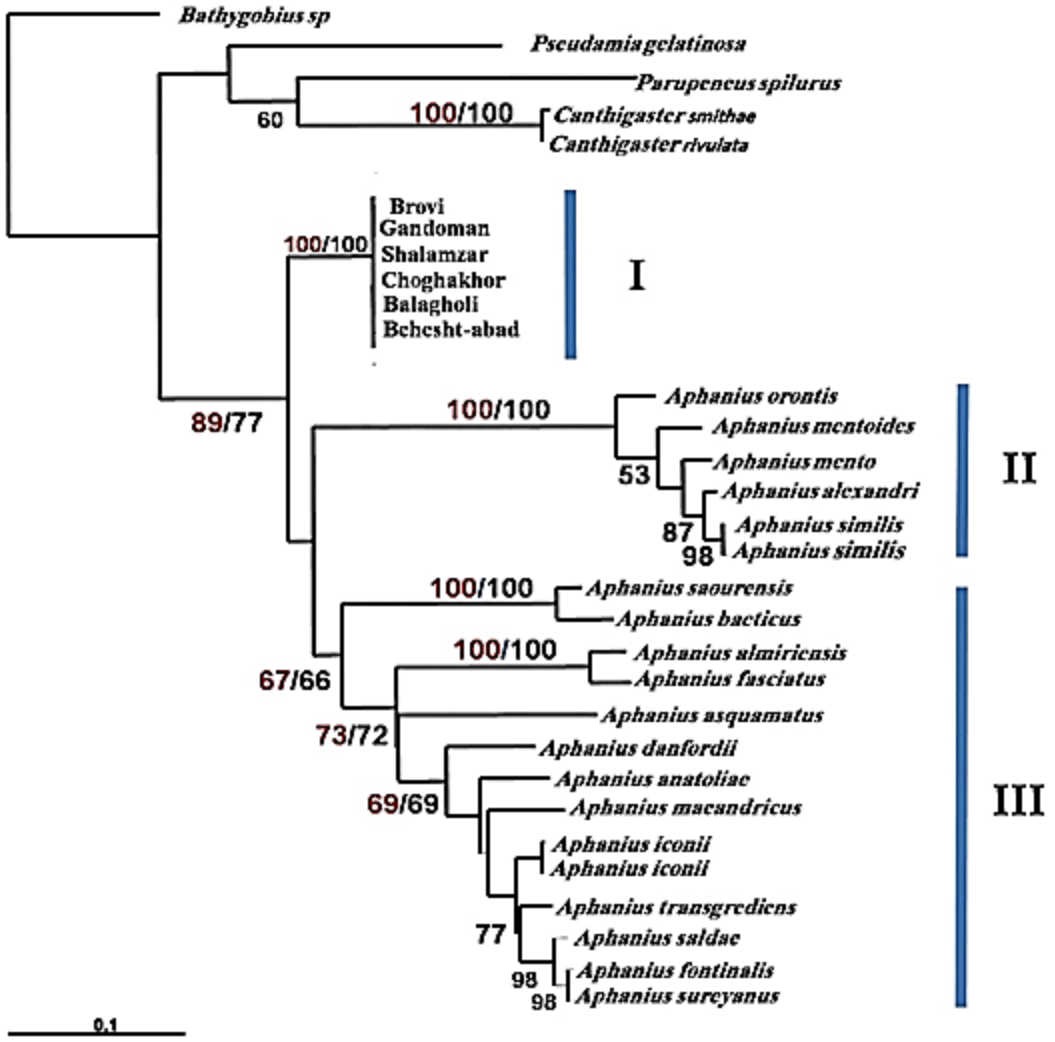

Figure 2. Phylogentic relationships of *Aphanius vladykovi* populations with other *Aphanius* spp. based on maximum likelihood and neighbor joining methods. Numbers along the branches are maximum likelihood and neighbor joining bootstraps values, respectively. The bootstrap support values of smaller than 50% are not shown. The blue bars denote the resolved groups.

vladykovi were in the ranges of intra-specific levels. Hence, all studied *Aphanius* specimens in the current study are *A. vladykovi*.

The distribution of the most frequent haplotypes in the different localities is expectable (Hashemzadeh Segherloo et al., 2012 a), since these localities are in close proximity to one another in the same river drainage, hence population interchange among them during different climatic events like flooding or even via anthropogenic activities is likely. As indicated in previous studies a sample size of 5-7 individuals can trap a good representative haplotype diversity of each population (Hubert et al., 2008), accepting this notion we can infer that Shalamzar, Balagholi, and Beheshtabad populations with more haplotypes have higher genetic diversity compared to other populations studied here. Among the haplotypes identified here, haplotypes *A. vla*3 (Choghakhor) and *A. vla*5 (Shalamzar) are probably younger haplotypes, since they are peripheral on the network with no haplotypes radiating from them. This inference should be treated cautiously, since in this study we have not included other Iranian *Aphanius* species, which are probably phylogenetically closer to *A. vladykovi* compared to species used from GenBank. Because it is possible that inclusion of other *Aphanius* spp. from Iran or nearby regions would change the connection patterns in haplotype network and the noted peripheral haplotypes may be intermediate to other species. Based on what is seen on the haplotype network

A. vladykovi is highly diverged from other *Aphanius* spp. included here.

So far, conservation activities regarding *A. vladykovi* have been focused only on the Choghakhor Wetland (the type locality of this species). Based on the results, there are more than one population of the species in the studied region. These populations exist mostly in small springs with different extents of anthropogenic effects like habitat changes, pollution, and water exploitation. Each of these isolated populations can be considered as a gene pool for conservation of *A. vladycovi*. Hence, we propose to consider all these populations and their related habitats in any conservation-oriented plans.

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