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The First Complete Mitochondrial Genome Sequence in the Genus *Aphanius* (Teleostei)

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Abstract—In the present study, for the first time we isolated and characterized the complete mitochondrial genome sequence of the endangered Farsi killifish *Aphanius farsicus* by long polymerase chain reaction amplification, and primer walking methods. The circular mitogenome of *A. farsicus* consisting of 16 485 base pairs encodes 13 polypeptides (protein-coding genes), the 12S and 16S ribosomal RNAs, and 22 transfer RNAs and an 884 bp D-loop control region. These genes are ordered in the same way as most other vertebrates. The overall nucleotide composition of this genome was 27.09 for A; 27.87 for T; 16.89 for G; and 28.14% for C (GC content of 45%, and AT content of 55%). The genus *Aphanius* has already been in the family Cyprinodontidae. However, the name Aphaniidae has recently been proposed as valid family for the members of the genus *Aphanius* (the Western Palaearctic killifishes), while the family Cyprinodontidae is restricted to the New World genera such as *Cyprinodon*, *Floridichthys* and *Jordanella*. Based on the phylogenetic relationships achieved in the present study, we recommend that the validation of family Aphaniidae still needs more phylogenetic supports, and this can be investigated by adding more sequences of the *Aphanius* species. The availability of this mitogenome will also provide a set of useful data for studying on population genetic diversity and molecular evolution and facilitate evaluations of *A. farsicus* genetic structure for management and conservation of this endangered species.

Keywords: Aphanius, mitogenome, gene arrangement, phylogenetic relationship, population genetic diversity, Iran

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INTRODUCTION

The complete mitochondrial DNA (mtDNA) sequence of vertebrates is a circular molecule with a length of about 16–19 kb (Anderson et al., 1981; Boore, 1999). The mitochondrial genome is frequently used for phylogenetic studies and population genetic analyses because of its compressed gene organization, fast evolutionary rate, maternal inheritance and lack of genetic recombination compared with the nuclear genome (Avise, 1994; Miya et al., 2001).

In recent years, complete mitochondrial DNA sequences have been extensively used to reconstruct the phylogeny of higher-level taxa (e.g. Kim et al., 2004; Jondeung and Karinthanyakit, 2015; Quezada-Romegialli et al., 2015).

The genus *Aphanius* has already been placed in the family Cyprinodontidae. However, based on the outcomes of recent molecular studies (e.g., Pohl et al., 2015; Helmstetter et al., 2016; Reznick et al., 2017), *Aphanius* is more closely related to *Valencia*, the livebearers of the families Anablepidae and Poeciliidae, and the African lampeyes (*Aplocheilichthys* and related genera), than to other genera in Cyprinodontidae. Therefore, Freyhof et al. (2017) concluded that the family Cyprinodontidae, as proposed by Parenti (1981), is polyphyletic, and proposed Aphaniidae as a valid family name for genus *Aphanius* (the Western Palaearctic killifishes).

The members of genus *Aphanius* are widely distributed throughout the Old World (Western Palaearctic and Near East) (Nelson et al., 2016; Freyhof et al., 2017). These fishes are commonly known as killifish or tooth-carps (Wildekamp, 1993). Among vertebrates, the family Aphaniidae represents a group that is particularly useful to study the micro-evolutionary processes shaping patterns of genetic structure and of geographic variation in natural populations, or of speciation processes associated to unstable ecological conditions (Villwock, 1976; Ferrito et al., 2007).

The different *Aphanius* species inhabit wide range of coastal and landlocked habitats in the drainages of Mediterranean, Red Sea and the Persian Gulf basins (Widemakp, 1993). In its native distribution ranges, 34 species have been recorded for the genus *Aphanius* so far (Wildekamp, 1993; Teimori et al., 2016; Van der Laan and Fricke, 2018).

Fig. 1. Female (a) and male (b) of *Aphanius farsicus* captured from a live fish kept in aquarium. Photo provided by Mehregan Ebrahimi.

By considering its diversity and biogeographic distribution, central Anatolia and Iran are known to show the highest diversity of *Aphanius* members (Hrbek and Meyer, 2003; Teimori, 2013; Freyhof et al., 2017; Teimori et al., 2018). Currently, 15 extant and one fossil *Aphanius* species have been discovered and reported from Iran (e.g. Coad, 2000, 2009; Hrbek et al., 2006; Gholami et al., 2014; Esmaeili et al., 2014; Teimori et al., 2012, 2014, 2018) in which 12 species are endemic to this country (Teimori et al., 2016). Therefore, the notable feature of Iranian killifish diversity is its high endemicity. Most of the endemic *Aphanius* species in this country are distributed in the closed endorheic basins associated with the mountainous regions of Zagros, and characterized by small population size (Gholami et al., 2014; Teimori et al., 2016).

Aphanius farsicus (Fig. 1) is an endemic killifish in the endorheic Lake Maharlu Basin, Southern Iran (Teimori et al., 2011; Esmaeili et al., 2016). It is found in freshwater streams and springs and in the springs of varying saline content or saline influence from hypersaline chloride Lake Maharlu (Esmaeili et al., 2016).

Several studies have used mtDNA to study genetic diversity and phylogeny and evolution of *Aphanius* species so far. Yaripour et al. (2017) by applying five microsatellite markers studied genetic structure of *Aphanius* farsicus from the Maharlu Lake basin in Southern Iran. Another study by Salimi et al (2018) investigated genetic diversity in two *Aphanius* species (*A. ginaonis* and *A. stoliczkanus*) in southern Iran by using PCR-RFLP technique. Angeletti et al. (2010) studied genetic diversity of *Aphanius fasciatus* and found that the genetic variability of this species

Table 1. Primer pairs used to amplify mtDNA of *Aphanius farsicus* with long-PCR (all of the primers are designed in the present study)

No. of fragment	Primer name	Nucleotide sequence (5'–3') and location	Product length	Annealing *T*,°C
1	AF-S1F	176 CTGGTATCAGGCACGCCTTTG 196	3508	57
	AF-S1R	3665 CTGTGGCAATAAGGGCGAGG 3684		
2	AF-S2F	3660 CAACACCTCGCCCTTATTGC 3679	3310	55
	AF-S2R	6950 CTCCACGTTAGTGGCTGTTAA 6970		
3	AF-S3F	6872 TCCCTAGTAGCCGTAATCATGTTCC 6896	4021	57
	AF-S3R	10873 GCTTTGGTATGTCAGGAGGGC 10893		
4	AF-S4F	10869 TGTAGCCCTCCTGACATACCAAAG 10892	2660	58
	AF-S4R	13505 GGAGGTGAGTGAACGAAGTTGTAGG 13529		
5	AF-S5F	13504 CCCTACAACTTCGTTCACTCACCTC 13528	1896	58
	AF-S5R	15379 GGAACTGTGTTACGGGACGGAA 15400		
6	AF-S6F	15375 AACCTTCCGTCCCGTAACACAG 15396	1341	58
	AF-S6R	190 GGTGTTATGGGCTGCAAAGGC 210		

strongly reduced through time. Also, several previous studies (e.g. Hrbek and Meyer, 2003; Teimori et al., 2012, 2013, 2014, 2018; Esmaeili et al., 2014) investigated phylogenetic relationships, and evolutionary history of different *Aphanius* species and populations. Therefore, these studies show the value of mtDNA markers for population genetic structure, stock identification, phylogeny, evolution and conservation genetics.

For that reason, in this study, for the first time we sequenced, and characterized the complete mtDNA genome of the endangered killifish *A. farsicus*. Additionally, we conducted phylogenetic analyses based on the complete mitochondrial genome with the purpose of investigating the phylogenetic position of *A. farsicus* within the order Cyprinodontiformes.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

The sample of *Aphanius farsicus* was collected by hand net from a small spring around the Lake Maharlu in Southern Iran. The species were identified at the field by observing morphological characters. A piece of muscle tissue was removed from the individual and preserved in 95% ethanol for DNA extraction. Total genomic DNA from muscle tissue was extracted using standard protocol of *FavorPrep Tissue Genomic DNA Extraction Mini Kit* (FAVORGEN).

Primers Design and Sequencing

The complete mitogenome of *A. farsicus* was amplified using a long polymerase chain reaction amplification (long PCR-technique) (Miya and Nishida, 1999; Miya and Nishida, 2000). Six sets of primers (Table 1) were designed based on multiple alignments of the conserved region of the complete mitochondrial DNA sequences of other Cyprinodontiformes extracted from NCBI; including *Orestias ascotanensis* (NC_027582) (Quezada-Romegialli et al., 2016), *Cyprinodon variegatus* (KR061357) (Barcelon and Lema, 2016) and *Jordanella floridae* (AP006778) (Setiamarga et al., 2008) as well as the partial sequence of 12S ribosomal RNA gene, the complete sequence of tRNA-Val gene, and the partial sequence of 16S ribosomal RNA gene which were previously determined for *Aphanius persicus* (AY593493) (Hrbek et al., 2006) (now *A. farsicus*, Teimori et al., 2011).

Thereafter, 25 normal PCR primer sets were designed to obtain contiguous, overlapping segments of the entire mitogenome (Table 2).

The NCBI Primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) used to design primers. Primers used in this study were synthesized by *DenaZist* and *Pishgam* companies (Tehran-Iran). It was necessary that every two contiguous segments overlapped by at least 50 bp to ascertain the accuracy of sequencing.

Mitochondrial DNA Amplification

Super PCR MasterMix 2X (Yekta *Tajhiz Azma-Tehran-Iran*) was used to amplify mitochondrial DNA segments using primers listed in Table 1. This reactions were carried out in 25 μl reaction mixture containing 12.5 μL of super PCR MasterMix 2X, 1 μL of DNA template (1 μg), 1 μL of each primer (10 μM), and 9.5 μl sterile distilled H_2O. These reactions consisted of an initial denaturing step at 94°C for 4 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 55–58°C (the details of temperature indi-

Table 2. Primer pairs used for normal PCR amplification (all of the primers are designed in the present study)

No. of fragment	Primer name	Nucleotide sequence (5'—3') and location	Product length	Annealing *T*, °C
1	AF-Mt-p1-F	40 AAGATGAGCCCTAGAAAGTTCC 61	957	54
	AF-Mt-p1-R	976 CTTCCGGTACACTTACCATGTT997		
2	AF-Mt-p2-F	974 GTAACATGGTAAGTGTACCGGAAGG 998	853	58
	AF-Mt-p2-R	1804 GTGTGCTTGGAGAGAGGTTTAGTC 1827		
3	AF-Mt-p3-F	1802 GAGACTAAACCTCTCTCCAAGCAC 1825	667	56
	AF-Mt-p3-R	2446 CTAGGGTAACTCGGTTCGTTGATC 2469		
4	AF-Mt-p4-F	2444 CCGATCAACGAACCGAGTTACC 2465	853	57
	AF-Mt-p4-R	3277 CGTGGTTGTCGGGTTGAAACT 3297		
5	AF-Mt-p5-F	3225 GGCCTCATCCTCTCGACTTGAC 3246	459	58
	AF-Mt-p5-R	3665 CTGTGGCAATAAGGGCGAGG 3684		
6	AF-Mt-p6-F	3663 CACCTCGCCCTTATTGCCAC 3682	888	57
	AF-Mt-p6-R	4531 CGTGGTTGTCGGGTTGAAACT 4551		
7	AF-Mt-p7-F	4480 GCCTCATCCTCTCGACTTGAC 4500	720	56
	AF-Mt-p7-R	5179 CACTTCCGCTTAGGGCTTTGAA 5200		
8	AF-Mt-p8-F	5178 CTTCAAAGCCCTAAGCGGAAGTG 5200	906	58
	AF-Mt-p8-R	6060 GAGAGAAGGAGAAGAACGGCAGTAA 6084		
9	AF-Mt-p9-F	6059 ATTACTGCCGTTCTTCTCCTTCTC 6082	837	56
	AF-Mt-p9-R	6873 GGAACATGATTACGGCTACTAGGG 6896		
10	AF-Mt-p10-F	5939 CTCCACCTAGCAGCAATTTCATC 5961	957	54
	AF-Mt-p10-R	6874 GGAACATGATTACGGCTACTAGG 6896		
11	AF-Mt-p11-F	6872 TCCCTAGTAGCCGTAATCATGTTCC 6896	731	57
	AF-Mt-p11-R	7584 CTAGAAGCCGGAATTGGCCG 7603		
12	AF-Mt-p12-F	7573 GACCTTACTCCCGGCCAATTC 7593	737	57
	AF-Mt-p12-R	8280 GATCTGTTGGGTAAAGCGGGAA 8301		
13	AF-Mt-p13-F	8278 GTTTCCCGCTTTACCCAACAGATC 8301	824	58
	AF-Mt-p13-R	9096 GTGATAGAAGGCTCAGAAGAATCCG 9120		
14	AF-Mt-p14-F	9045 CCTCCGTTACGGCATGATTC 9064	964	55
	AF-Mt-p14-R	9989 CACTGGAGACCTCCTTGAAGT 10009		
15	AF-Mt-p15-F	9994 AAGGAGGTCTCCAGTGAGCAG 10014	684	57
	AF-Mt-p15-R	10656 GAGAGATGTAAGTTCGTTGGCGG 10678		
16	AF-Mt-p16-F	10227 AACTCTTCAGCTCGACTCAAC 10247	666	54
	AF-Mt-p16-R	10874 GCTTTGGTATGTCAGGAGGG 10893		
17	AF-Mt-p17-F	10935 CCTGATGCACCTTCACTGCTG 10955	831	57
	AF-Mt-p17-R	11747 CAGGCAGTTCAGGGTCACAG 11766		
18	AF-Mt-p18-F	11745 TACTGTGACCCTGAACTGCCTG 11766	724	58
	AF-Mt-p18-R	12450 CAGCCAATGAGGAGGAAGGC 12469		
19	AF-Mt-p19-F	12450 GCCTTCCTCCTCATTGGCTG 12469	701	57
	AF-Mt-p19-R	13130 GGTGCCAGTTAGAGCTAGGCTA 13151		
20	AF-Mt-p20-F	13127 TCCTAGCCTAGCTCTAACTGG 13147	402	54
	AF-Mt-p20-R	13510 GGAGGTGAGTGAACGAAGTT 13529		
21	AF-Mt-p21-F	13504 CCCTACAACTTCGTTCACTCACCTC 13528	704	58
	AF-Mt-p21-R	14225 GCTAGATGCAGGGATGGGCT 14244		
22	AF-Mt-p22-F	14132 CCGAATATGCAAACACCACCTG 14153	874	56
	AF-Mt-p22-R	14958 GAAATGGAATGCGAAGAACCGG 14979		

Table 2. (Contd.)

No. of fragment	Primer name	Nucleotide sequence (5'—3') and location	Product length	Annealing *T*, °C
23	AF-Mt-p23-F	14869 ACAAACCTCCTATCCGCTGTCC 14890	531	57
	AF-Mt-p23-R	15380 GGAACTGTGTTACGGGACGGA 15400		
24	AF-Mt-p24-F	15375 AACCTTCCGTCCCGTAACACAG 15396	810	58
	AF-Mt-p24-R	16163 CCATTAGAGAGAACGCTCGGCAT 16185		
25	AF-Mt-p25-F	15961 GTAGTAAGAGACCACCATCAGTTG 15984	235	56
	AF-Mt-p25-R	190 GTGTTATGGGCTGCAAAGGC 209		

cated in Table 1) for 45 s and extension at 72°C for 3 min, following with final extension at 72°C for 7 min.

Six mitochondrial DNA segments with ranges from 1.3 to 4 kbp amplified and used as a template in normal PCR using primers listed in Table 2. The Taq DNA Polymerase MasterMix (*Ampliqon*) used for normal PCR using primer listed in Table 2.

The normal PCR was performed following the standard procedure; containing 12.5 μL of Taq DNA Polymerase MasterMix, 1 μL of amplified mitochondrial segment (achieved by the above mentioned process), 0.5 μL of each primer (10 μM), and add sterile distilled H_2O to bring volume to 25 μL. Normal PCR reactions consisted of an initial denaturing step at 94°C for 3 min, following 30 cycles of denaturing at 94°C for 30 s, annealing at 54–58°C (exact temperature indicated in Table 2) for 45 s and extension at 72°C for 1 min, following with final extension at 72°C for 5 min.

Negative controls were included in all PCR amplifications to confirm the absence of contaminants. PCR products were purified sequencing was accomplished by primer walking method (*Bioneer-south Korea*).

Sequence Editing and Analysis

Sequence trace files were corrected and aligned with the SeaView v. 3.2 (Gouy et al., 2010) and Geneious v. 11.1.5 (Kearse et al., 2012). The locations of 13 protein-coding genes and two rRNA genes were determined by their similarity to published mitogenomes of other killifish species as shown in Table 3, whereas the tRNA genes were identified using the program tRNAscan-SE 2.0 (Lowe and Chan, 2016). Some tRNA genes that could not be found by the tRNAscan-SE, were identified by their secondary structure and their position in the mitogenome (Zhang et al., 2009).

The structure of control region and its conserves motifs were identified by making a comparison with homologous sequences of reported teleost (Quezada-Romegialli et al., 2015; Keepers et al., 2016; Xu et al., 2018).

Phylogenetic Analyses

To clarify the phylogenetic position of *A. farsicus* within the order Cyprinodontiform, the complete mitochondrial genomes of other 15 killifish species were downloaded from GenBank database (Table 3). In this data set, possible close outgroups in Beloniformes were also choose to root phylogenetic trees (Table 3). We did not include gene ND6 in our data set for phylogenetic analysis owing to its high heterogeneity and poor phylogenetic performance (Miya and Nishida, 2000).

A final data set of 16530 bp including various Cyprinodontiform sequences accompany with two out groups was aligned using Muscle 3.6 (Edgar, 2004) as incorporated in SeaView, under default settings. Finally, phylogenetic position of *A. farsicus* based on 16 representative killifish complete mitogenome was validated by constructing a maximum likelihood tree with RAxML 7.2.8 (Stamatakis et al., 2008) under a GTR + G + I model, and 1000 rapid bootstrap replicates. The best-fit nucleotide substitution model (GTR + G + I) was selected by AIC (Akaike Information Criterion) using JModelTest (Posada, 2008).

RESULTS AND DISCUSSION

This is the first study that reports complete mitogenome of the genus *Aphanius*. The complete mitochondrial genome of *Aphanius farsicus* was 16.530 bp in size, including 13 protein-coding genes, 22 tRNA genes, two rRNA genes, and a putative control region. The gene order of *A. farsicus* mitochondrial genome was shown in Fig. 2, and the details of organization of the *A. farsicus* mitogenome are shown in Table 4. There was a non-coding region spanning 884 bp between genes $tRNA^{Phe}$ and $tRNA^{Pro}$ with a high A + T content that was identified as a putative control region (Table 4). The complete genome sequence was deposited in GenBank database under the accession number MN578038.

The gene order of mitochondrial DNA of *A. farsicus* was similar to other killifishes such *Orestias ascotanensis* (NC_027582), *Cyprinodon nevadensis amargosae* (KU883631) and *Jordanella floridae* (AP006778)

Table 3. Overview on the fish species used in this study

Order, Family	Species	Accession number	Reference
Cyprinodontiformes			
Rivulidae	*Kryptolebias marmoratus*	NC_003290	Lee et al., 2001
	K. marmoratus	AF283503	
	K. marmoratus	AF283503	
	K. hermaphroditus	KX268503	Kim et al., 2016
Aplocheilidae	*Aplocheilus panchax*	NC_011176	Setiamarga et al., 2008
Fundulidae	*Fundulus olivaceus*	AP006776	
Poeciliidae	*Poecilia sphenops*	NC_026579	Jiang et al., 2016
Goodeidae	*Xenotoca eiseni*	NC_011381	Setiamarga et al., 2008
	Orestias ascotanensis	NC_027582	Quezada-Romegialli et al., 2015
	Orestias sp.	KR_363189	
	Jordanella floridae	AP006778	Setiamarga et al., 2008
	J. floridae	NC_011387	
Cyprinodontidae	*Cyprinodon diabolis*	KX061747	Lema et al., 2016
	C. nevadensis	KU883631	
	C. tularosa	NC_028292	
	C. variegatus	KT288182	
Aphaniidae	*Aphanius farsicus*	MN578038	This study
Beloniformes			
Belonidae	*Ablennes hians*	AB373007	Setiamarga et al., 2008
Adrianichthyidae	*Oryzias dancena*	AB498069	Setiamarga et al., 2009

(Setiamarga et al., 2008; Quezada-Romegialli et al., 2016).

The overall nucleotide composition of mitochondrial genome in *A. farsicus* was 27.09 for A; 27.87 for T; 16.89 for G; and 28.14% for C (GC content of 45%, and A + T content of 55%). The overall GC and AT content in *A. farsicus* was almost close to the fishes of family Cyprinodontidae such as *Orestias ascotanensis, Jordanella floridae and Cyprinodon diabolis* rather than the members of other families of the order Cyprinodontiformes (Table 5). These species are phylogenetically close to *A. farsicus* (Aphaniidae) rather than other examined fishes of the order Cyprinodontiformes (see phylogenetic tree).

Protein-coding Gene (PCG) Features

Aphanius farsicus mitochondrial genome encoded 13 protein-coding genes with 11462 bp in length, which accounted for 69.34% of the complete mitogenome (Table 5).

The boundaries between protein-coding genes of the mitochondrial genome were determined by aligning their sequences and identifying translation initiation and termination codons with comparison to other killifishes. The nucleotide composition in 13 protein coding genes was 25.40 for A, 30.27 for C, 15.43 for G and 28.88% for T. The A+T content of protein-coding gene were 61.20%. The lengths of protein-coding genes ranged in size from 169 (ATP8) to 1863 bp (ND5).

Transfer and Ribosomal RNA Gene Features

The complete mitochondrial genome in *Aphanius farsicus* contained 22 tRNA genes, which were totally 1552 bp in length and interspersed between the rRNA and the protein-coding genes with the ranges from 65 bp ($tRNA^{Try}$) to 77 bp ($tRNA^{Ser}$ and $tRNA^{Pro}$). The overall A + T content of 22 tRNAs was 57.20% with the composition of A = 30.21, G = 20.61, T = 26.99, C = 22.16%. The 16S and 12S ribosomal RNA genes were 1687 and 945 bp in length respectively, and located between genes $tRNA^{Leu}$ and $tRNA^{Phe}$ and separated by gene $tRNA^{Val}$. The A + T content was 55.80% for 16S rRNA gene (A = 32.36, G = 21.57, T = 23.41, C = 22.64%), and 52.20% for 12S rRNA gene A = 31.21, G = 21.26, T = 20.95, C = 26.56%), respectively.

Non-Coding Control Region (CR)

The nucleotide composition in D-loop (non-coding control region) was 28.95 for A, 23.19 for C, 15.61 for G and 26.58% for T. The A + T content of the control region were 61.20%, which was higher than the

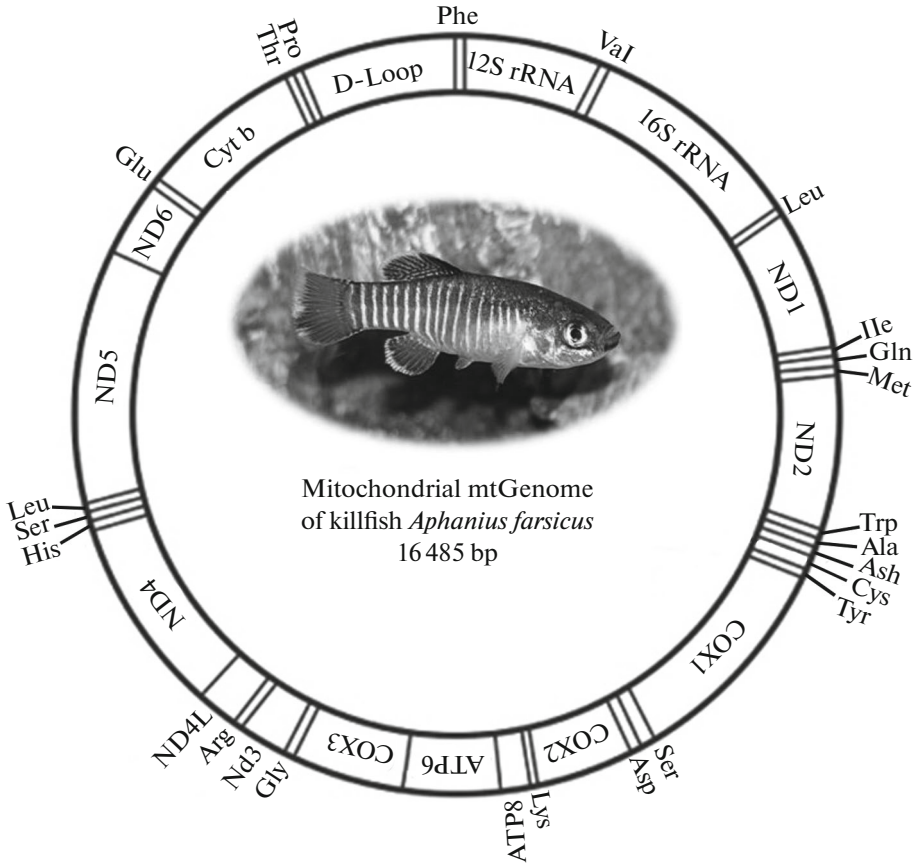

Fig. 2. Gene organization of *Aphanius farsicus* mitochondrial DNA. *ND1-6* refers to NADH dehydrogenase subunits 1-6. *COI-III* refers to cytochrome c oxidase subunits 1-3, *ATP6* and *ATP8* refers to ATPase subunits 6 and 8, and Cyt *b* refers to *cytochrome* b.

average value of the whole mitochondrial genome (i.e., 55.00%) (Table 4).

The mitochondrial DNA control region is a variable sequence that have a regulatory functional in transcription and replication of mtDNA. It is characterized by conserved sequence blocks, including the typical tripartition with a terminal associated sequence (TAS), a central and conserved sequence block (CSB) domains containing the conserved sequence blocks CSB-F, CSB-E and CSB-D (Sbisà et al., 1997). It additionally has a variable domain consists of three conserved sequence blocks (CSB-1, CSB-2, CSB-3). These blocks can be determined by multiple homologous sequence alignment with other related taxa.

The TAS domain is located between the 5' end of the control region and the beginning of the CSB-F. Generally, the length of TAS domain varied from 243 bp to 357 bp (Wang et al., 2011). Our results indicate that the TAS domain in *A. farsicus* was 301 bp length, and started with TACAT (Fig. 3). In addition, two central conserved sequence blocks CSB-F (TGAGACAAAAATCGTGGGGG) and CSB-D (TATTACTGGCATTTGGTTCCT) were detected in the control region of *A. farsicus* mitogenome (Fig. 3), while the typical central conserved CSB-E could not be found. Moreover, conserved sequence blocks; CSB-1, CSB-2 and CSB-3 were identified at the 3' end of the control region. These sequences might be associated in positioning RNA polymerase (Clayton, 1991; Shadel and Clayton, 1997).

Phylogenetic Analysis

Maximum likelihood tree was estimated based on whole mitochondrial genome of 16 examined killifish including *Aphanius farsicus* (Fig. 4). The ML tree presented seven major clades corresponding to the families of the order Cyprinodontiformes i.e., Rivulidae, Aplocheilidae, Poeciliidae, Fundulidae, Goodeidae, Cyprinodontidae, and Aphaniidae. Phylogenetic relationships based on the whole mitogenome of the examined killifishes are congruent with previous work (Quezada-Romegialli et al., 2015).

All the studied families are monophyletic. However, phylogenetic position of the clade VI (family Cyprinodontidae) and VII (family Aphaniidae) are questionable. In their previous study, Quezada-Romegialli et al. (2015) have documented high intra-family variability in the family Cyprinodontidae with regard to the whole mitogenome. They showed that

Table 4. Organization of the *Aphanius farsicus* mitogenome

Gene	Position from	Position to	Size, bp	Base composition, number				C + G content, %
				A	T	G	C	
$tRNA^{Phe}$	1	66	66	27	16	14	11	36.8
12S rRNA	67	1011	945	295	198	201	251	47.8
$tRNA^{Val}$	1012	1083	72	24	16	15	17	44.4
16S rRNA	1084	2770	1687	546	395	364	382	44.2
$tRNA^{Leu}$	2771	2844	72	19	19	17	19	48.6
ND1	2845	3891	1045	288	288	145	326	45.0
$tRNA^{Ile}$	3892	3959	66	20	15	18	15	48.5
$tRNA^{Gln}$	3960	4029	68	23	17	12	18	42.9
$tRNA^{Met}$	4030	4098	69	21	21	11	16	39.1
ND2	4099	5145	1047	288	288	145	326	45.0
$tRNA^{Trp}$	5146	5216	71	24	11	17	19	50.7
$tRNA^{Ala}$	5217	5285	69	20	24	15	10	36.2
$tRNA^{Asn}$	5286	5358	73	16	25	19	13	43.8
$tRNA^{Cys}$	5359	5424	66	19	15	16	16	48.5
$tRNA^{Tyr}$	5425	5490	66	17	23	16	10	39.4
COI	5491	7043	1553	355	467	285	446	47.1
$tRNA^{Ser}$	7044	7120	77	23	18	16	20	46.8
$tRNA^{Asp}$	7121	7192	72	19	18	17	18	48.6
COII	7193	7882	690	194	186	108	203	45.0
$tRNA^{Lys}$	7883	7955	73	23	14	15	21	49.3
ATPase8	7956	8124	169	49	43	20	57	45.6
ATPase6	8125	8808	684	160	202	94	228	47.1
COIII	8809	9596	788	181	221	139	244	48.8
$tRNA^{Gly}$	9597	9639	43	26	22	10	14	33.3
ND3	9640	9987	348	60	123	63	103	47.6
$tRNA^{Arg}$	9988	10056	69	25	24	10	10	29.0
ND4L	10057	10353	297	61	81	47	108	52.2
ND4	10354	11733	1380	335	408	211	428	46.2
$tRNA^{His}$	11734	11801	68	25	22	11	10	30.9
$tRNA^{Ser}$	11802	11869	68	13	15	21	19	58.8
$tRNA^{Leu}$	11870	11941	72	22	19	12	19	43.1
ND5	11942	13815	1874	504	577	254	528	42.0
ND6	13816	14322	507	154	90	90	183	52.4
$tRNA^{Glu}$	14323	14390	68	22	18	10	18	41.2
Cytb	14391	15463	1073	279	337	168	290	42.6
$tRNA^{Thr}$	15464	15540	77	21	29	11	16	35.1
$tRNA^{Pro}$	15541	15610	70	20	18	17	15	45.7
Control Region (CR)	15611	16485	883	256	285	138	205	38.8

Table 5. Overview on nucleotide composition of the mitogenome in *Aphanius farsicus* and other killifish species used in this study

Family	Species	Content, %						Reference
		A	G	T	C	GC	A + T	
Rivulidae	*Kryptolebias marmoratus*	27.52	15.81	29.15	27.50	43.70	56.70	Kim et al., 2016
Aplocheilidae	*Aplocheilus panchax*	29.23	15.79	28.05	26.92	42.70	57.30	Setiamarga et al., 2008
Fundulidae	*Fundulus olivaceus*	27.94	16.10	29.84	28.08	42.20	57.80	
Poeciliidae	*Poecilia sphenops*	29.44	15.55	27.15	28.84	43.40	56.60	Jiang et al., 2016
Goodeidae	*Xenotoca eiseni*	29.23	14.81	29.25	26.69	41.50	58.50	Setiamarga et al., 2008
Cyprinodontidae	*Orestias ascotanensis*	26.68	17.03	29.15	27.11	44.20	55.80	Quezada-Romegialli et al., 2015
	Orestias sp.	26.71	17.03	29.15	27.08	44.10	55.90	
	Jordanella floridae	27.14	16.09	27.86	28.90	45.00	55.00	Setiamarga et al., 2008
	Cyprinodon diabolis	25.69	17.48	27.31	29.50	47.00	53.00	Lema et al., 2016
	C. nevadensis	25.60	17.52	27.36	29.50	47.02	52.96	
	C. tularosa	26.01	17.20	27.09	29.68	46.88	53.10	
	C. variegatus	26.33	16.89	27.21	29.55	45.66	53.54	
Aphaniidae	*Aphanius farsicus*	27.09	16.89	27.87	28.14	45.00	55.00	This study

within the family Cyprinodontidae, the genus *Orestias* is monophyly with high bootstrap value, and is sister to a clade containing *Jordanella* and *Cyprinodon*. The monophyly of genus *Orestias* and its phylogenetic relation to the genera *Jordanella* and *Cyprinodon* are now supported by our phylogenetic analysis (Fig. 4). However, they did not analyze genus *Aphanius* in their study.

Here for the first time we provided the complete mitogenome in one of the *Aphanius* species i.e., *A. farsicus*. In our phylogenetic hypothesis, we included the complete mitogenome of *A. farsicus* into the data set,

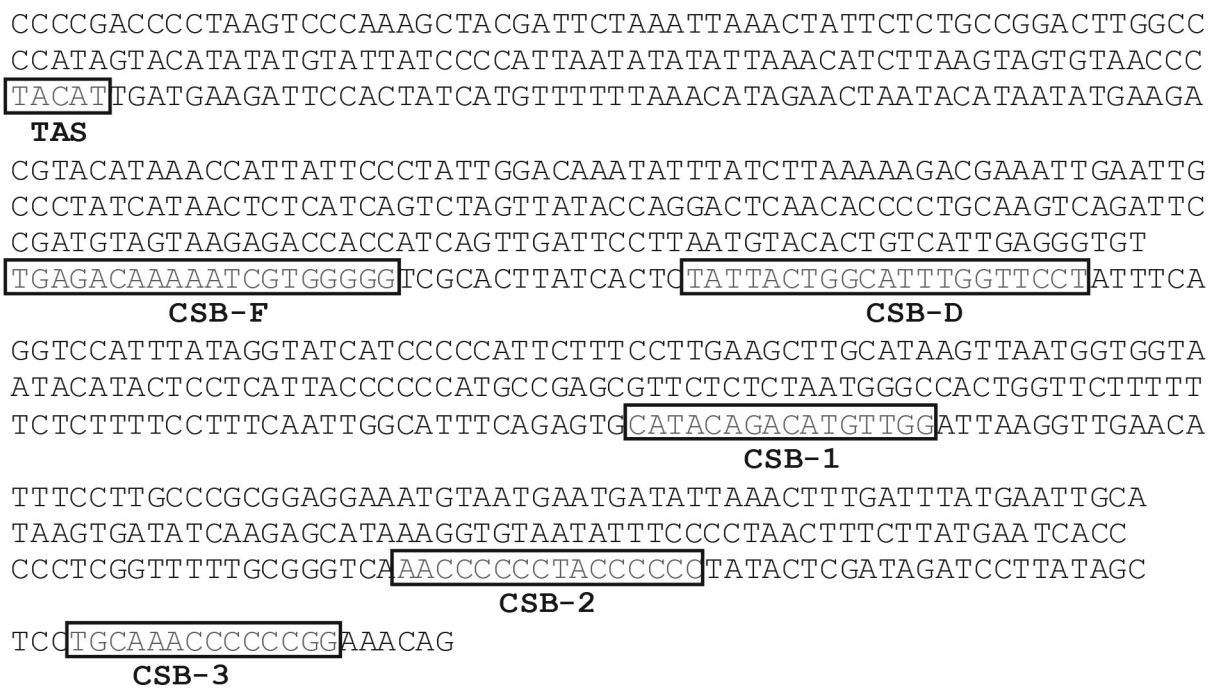

Fig. 3. Partial sequence of mitochondrial control region of *Aphanius farsicus*. In the control region, the termination associated sequence (TAS), central conserved sequence blocks (CSB-F, CSB-D), and conserved sequence blocks (CSB-1, CSB-2 and CSB-3) are boxed and marked.

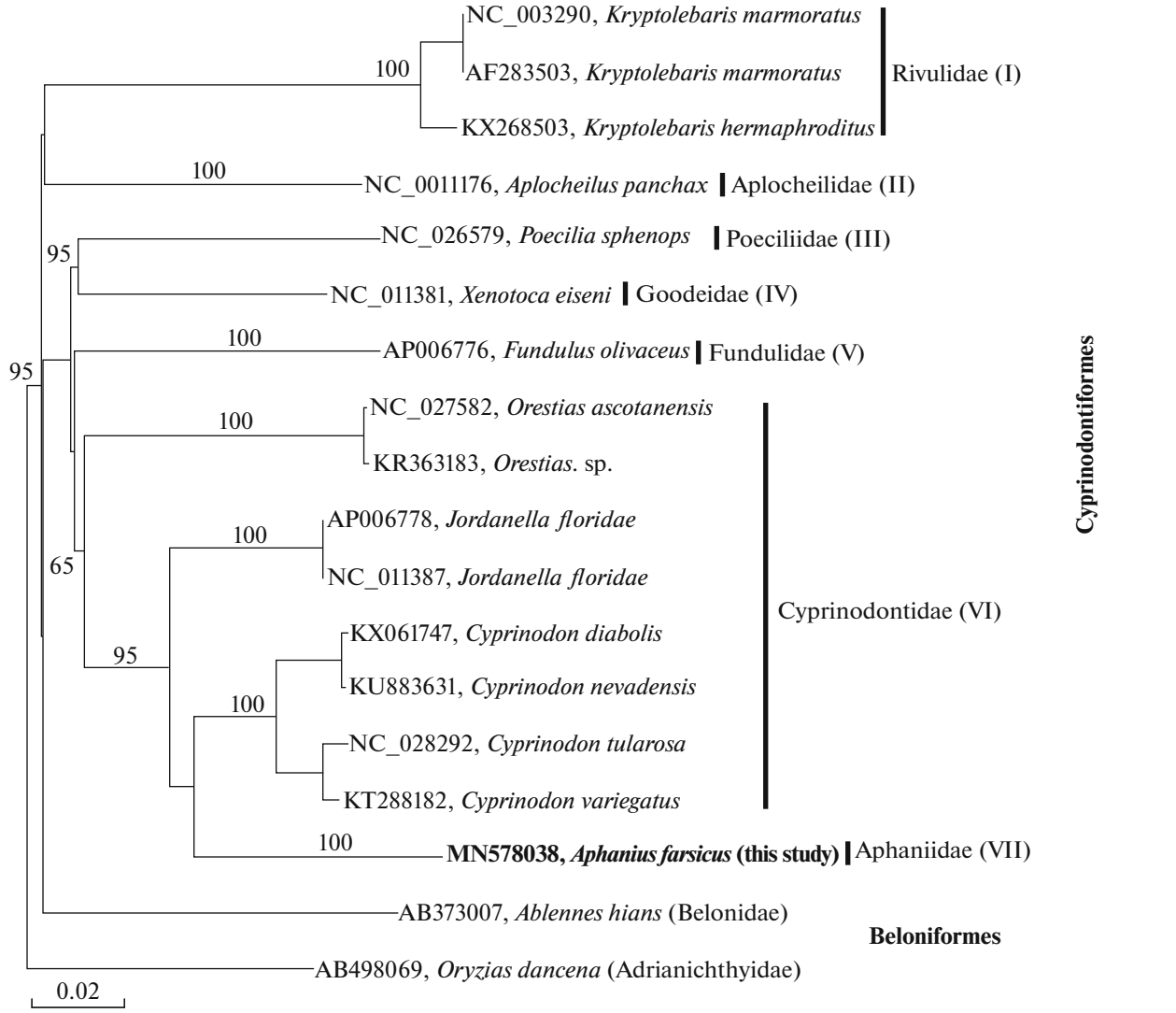

Fig. 4. Phylogenetic hypothesis of *Aphanius farsicus* and related taxa in the order of Cyprinodontiformes based on their complete mitogenome. On each branch is denoted maximum likelihood bootstrap support. Numbers behind the species refer to the accession numbers.

and found that the genus *Cyprinodon* is sister to the genus *Aphanius* and both together form sister group to the genus *Jordanella*.

It should be noted that the genus *Aphanius* has already been in the family Cyprinodontidae. However, in their recent study, Freyhof et al. (2017) proposed the name Aphaniidae as valid family for the members of the genus *Aphanius* (the Western Palaearctic killifishes), while restricting the family Cyprinodontidae to the New World genera such as *Cyprinodon*, *Floridichthys* and *Jordanella*.

As mentioned above, in our phylogenetic analysis, a close phylgenetic relation was found between the genera *Aphanius* (now in the family Aphaniidae) and *Cyprinodon* (now in the family Cyprinodontidae) considering their complete mitogenome. Therefore, we assumed that the validation of family Aphaniidae still needs more phylogenetic supports, and this can be investigated by adding more sequences of the *Aphanius* members.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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