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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Genetic analysis of Aphaniidae Hoedeman, 1949 (Teleostei: Cyprinodontiformes) family in Anatolia[*]

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Abstract: We tested the mitochondrial DNA cytochrome b gene-based (1065 bp.) phylogeny and genetic diversity of tooth-carp fish (Aphaniidae Hoedeman, 1949), many of which are endemic, with a very high species diversity in Anatolia. A total of 107 individuals were studied from 29 stations belonging to 19 Aphaniid species and forty-four haplotypes were identified, all of which were species-specific.

The phylogenetic relationships generated by neighbor joining, maximum likelihood and maximum parsimony methods are fully compatible with each other. The generally well supported phylogenetic tree results and genetic distance results supported a structure divided into four lineages corresponding to four genera (*Anatolichthys*, *Paraphanius*, *Aphanius*, and *Kosswigichthys*). The genetic distance between these four lineages indicated a significant value ranging from 16.6% (between *Aphanius* and *Anatolichthys*) to 23.1% (between *Aphanius* and *Paraphanius*). Interspecies genetic distances ranged from 1.9% (between *P. boulengeri* and *P. similis*) to 24.52% (between *A. villwocki* and *P. mentoides*), except for two interspecies distances (*A. fontinalis – A. sureyanus*, 0.13% and *A. maeandricus – A. irregularis*, 0.57%).

Our results agree with previous studies of the Anatolian Aphaniidae family, which showed a diversification pattern shaped by Pliocene orogenic events. The present results indicate that mitochondrial DNA cytochrome *b* gene sequences are effective for Aphaniidae species identification and phylogenetic analysis.

Keywords: Anatolia, cytochrome b, phylogeny, tooth-carp.

Anadolu'daki Aphaniidae Hoedeman, 1949 (Teleostei: Cyprinodontiformes) ailesinin genetik analizi

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Öz: Anadolu'da çok yüksek tür çeşitliliğine sahip, çoğu endemik olan dişli sazancık balıklarının (Aphaniidae Hoedeman, 1949) mitokondri DNA sitokrom b genine (1065 bç.) dayalı filogenisini ve genetik çeşitliliğini test ettik. 19 Aphaniid türüne ait 29 istasyondan toplam 107 örnek incelendi ve türe özgü olan 44 haplotip tanımlandı.

Komşu birleştirme, maksimum olabilirlik ve maksimum tutumluluk yöntemleriyle oluşturulan filogenetik ilişkiler birbiriyle tam uyumlu sonuçlar verdi. Genel olarak iyi desteklenen filogenetik ağaç sonuçları ve genetik uzaklık sonuçları, dört cinse (*Anatolichthys*, *Paraphanius*, *Aphanius*, and *Kosswigichthys*) karşılık gelen dört soydan oluşan bir yapıyı destekledi. Bu dört soy grubu arasındaki genetik mesafe %16,6 (*Aphanius* ve *Anatolichthys*) ile %23,1 (*Aphanius* ve *Paraphanius*) arasında değişen ciddi bir değer gösterdi. Türler arası genetik mesafeler iki tür grubu (*A. fontinalis – A. sureyanus*: %0.1 ve *A. maeandricus – A. irregularis*: %0.6) dışında %2.8 (*P. boulengeri* ve *P.similis*) ile %24.5 arasında (*A. villwocki* ile *P. mentoides*) arasında değişmektedir.

Sonuçlarımız aynı zamanda, Pliyosen orojenik olaylarıyla şekillenen bir çeşitlenme modeli gösteren Anadolu Aphaniidae familyasının önceki çalışmalarıyla da uyumludur. Mevcut sonuçlar, mitokondriyal DNA sitokrom b gen dizilerinin Aphaniidae türlerinin tanımlanması ve filogenetik analizi için etkili olduğunu göstermektedir.

Anahtar kelimeler: Anadolu, dişli sazancık, filogeni, sitokrom b.

[*] *This study was produced from the master thesis.*

INTRODUCTION

Members of the family Aphaniidae Hoedeman, 1949, known as tooth-carp fish, are naturally distributed in coastal (brackish and euryhaline waters) and inland waters (creeks, streams, rivers, lakes, ponds, and marshes). The aphaniids are distributed in the coastal regions of the Mediterranean, the Gir Peninsula of northwestern India, the Red Sea, and Persian Gulf, and northeastern Somalia, and are among the most species-rich families in the order Cyprinodontiformes (Wildekamp, 1993; Coad, 2000; Gholami et al., 2014; Reichenbacher et al., 2007; Teimori et al., 2018; Esmaeili et al., 2020). The highest species diversity in aphaniids is found in the Central Anatolian and Iranian plateau (Coad, 2000). In fact, the Central Anatolian geography is considered the center of diversity of the aphaniids (Wildekamp et al., 1999). Members of the aphaniids are relict species and are considered to be the oldest known secondary freshwater fishes in Anatolia (Hrbek & Meyer, 2003). The distribution area of the family coincides with Tethys and Paratethys covering most of Europe from the early Eocene to Miocene (Hrbek & Meyer, 2003). The tooth-carp fish is considered to be a remnant of the Tethys, thought to have evolved from a common ancestor scattered around the Tethys Sea (Kosswig, 1967; Por & Dimentman, 1989). This hypothesis is supported by molecular analysis of mitochondrial DNA genes (Hrbek & Meyer, 2003).

Members of the Aphaniidae thought to have diversified due to geological events in the ancient Tethys region and the effect of ecological factors, were defined within a single genus *Aphanius* until recent studies. Hrbek et al. (2002), and Hrbek and Meyer (2003) mentioned the presence of six sublineages in analyses based on mitochondrial DNA genes (12S, 16S ribosomal RNA, NADH I and II). Subsequently, Esmaeili et al. (2020) stated that three lineages should be identified as *Aphanius*, *Aphaniops* and *Paraphanius* based on DNA barcoding. Finally, Freyhof and Yoğurtçuoğlu (2020) suggested that monophyletic species groups that emerge in phylogenetic analyses should be evaluated together with morphological characters and defined into eight lineages (*Anatolichthys*, *Kosswigichthys*, *Aphanius*, *Aphaniops*, *Paraphanius*, *Tellia*, *Esmaeilius* and *Apricaphanius*). Today, the aphaniids are represented by eight genera.

Freyhof and Yoğurtçuoğlu (2020) had already reported that 21 species of aphaniids are distributed in Anatolia and many species of toothedcarp species are threatened. According to the Red List of Threatened Species of the International Union for Conservation of Nature (IUCN, 2021), ten Anatolian aphaniid species are listed. Three of them are critically endangered (CR), one species endangered (EN), one species nearly threatened (NT), one species extinct (EX), and four other least concern species (LC). In addition, eleven species were not evaluated (NE).

The aim of this study was to determine the genetic structure and phylogenetic relationships within and among Anatolian Aphaniidae species, most of which are endangered, using mtDNA cytochrome b gene sequences.

MATERIAL AND METHOD

Sampling: A total of 107 individuals from Aphaniidae distributed in Anatolia were sampled at 29 stations using electroshockers (Table 1). The collected samples were first anesthetized with tricaine methane sulfonate solution (MS222), then their species level were identified according to taxonomic keys and labeled, and fixed with 96% ethyl alcohol. Except for the DNA sequence analysis, all laboratory work was carried out in the genetics laboratory of the Faculty of Fisheries of Recep Tayyip Erdogan University.

DNA Extraction, PCR Amplification and DNA Sequencing: Genomic DNA was extracted from the ethanol-fixed fin clips using DNeasy Blood & Tissue Kit (Qiagen, USA) following the manufacturer's protocol carried out in the Qiacube Automated DNA purification system. The DNA concentration and purity of each sample were assessed by spectrophotometry (Nanodrop, 2000/c, Thermo Scientific, USA), while the integrity was assessed by 1% TAE-agarose gel electrophoresis containing 0.5 mg/l EtBr.

The vertebrate mtDNA cytochrome b (*cytb*) gene was amplified using L14724: 5'-GTGACTTGAAAAACCACCGTTG-3'; H15915: 5'-CAACGATCTCCGGTTTACA AGAC-3' primers (Anderson et al., 1981). PCR reactions were carried out in 50 µl total volume containing 5 µl of 10X reaction buffer; 5 µl $MgCl_2$ (25 mM); 8 µl of dNTPmix (10 mM); 1 µl of forward primer (10 pmol); 1 µl of reverse primer (10 pmol); 0.2 µl of Taq DNA polymerase (1 U); 3 µl of DNA template (50 ng/µl); and 26.8 µl sterilized pure water. PCR reactions were performed using a gradient thermal cycler Biorad T100™ (Bio-Rad, Hercules, USA). The PCR condition was as follows: 1 cycle at 95 °C for 3 min for initial denaturation, followed by 35 cycles denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, ended up with a final extension for one cycle at 72°C for 5 min. Assessment of concentrations and sizes of PCR products were performed both spectrophotometrically and by 1.2% TAE-agarose gel electrophoresis containing 0.5 mg/l EtBr. The amplicons were visualized on UV Quantum-Capt ST4 system (Vilber Lourmat, France).

PCR products were directly sequenced in both directions using the L14724 and H15915 primers on an ABI

3730XL DNA Analyzer (Applied Biosystems) by Macrogen Inc. (Amsterdam, The Netherlands).

Molecular Data Analysis: The vertebrate mtDNA *cytb* raw sequences were aligned by Clustal-W (Thompson et al., 1994) and edited manually with Bioedit 7.0.0 (Hall, 1999). The number of haplotype, haplotype and nucleotide diversity were calculated using the software DNASP v.5.10.01 (Librado & Rozas, 2009). The number of polymorphic sites, the nucleotid number of conserved, variable and parsimonic informative, the nucleotide composition, number of transitions and transversions were calculated using the MEGA version X (Kumar et al., 2018). Average inter-specific and intra-specific, average genetic distances were calculated using Kimura two-parameter model (K2P; Kimura, 1980) implemented in MEGA version X (Kumar et al., 2018).

Phylogenetic analyses were performed by using neighbour joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) analyses. NJ trees were generated using MEGA X (Kumar et al., 2018) with 1000 bootstrap replicates according to K2P+G method. MP analysis was performed using PAUP* 4.0b10 (Swofford, 2003) with heuristic search, TBR branch-swapping, 1000 bootstrap replicates (Felsenstein, 1985), random addition sequence with 10 replicates. According to the Akaike information criterion (AIC), jModeltest v.0.1.1 (Posada, 2008) selected the TN93+G+I as the best model evolution for the *cytb* dataset. ML analysis with 100 bootstrap replicates implemented in PhyML ver. 2.4.4 (Guindon & Gascuel, 2003). To evaluate the evolutionary relationships of Aphaniidae species, *Cyprinodon variegatus* (GenBank accession number: NC028088) was used as outgroup for rooting.

RESULTS

A total of 107 Aphaniidae specimens were collected from 29 stations during the field studies. A total of 19 species belonging to four genera (*Aphanius*, *Kosswigichthys*, *Anatolichthys* and *Paraphanius*) from the Aphaniidae family were identified based on morphological diagnostic keys. Information about the study material is given in Table 1. From Aphaniidae genera in Anatolia, *Aphanius* and *Kosswigichthys* are represented by one species, while *Anatolichthys* and *Paraphanius* are represented by 12 and 5 species, respectively.

Table 1. Sampling and location information with haplotype code.

Species	Locations	Coordinates	Sample Number	Haplotype Code
Aphanius fasciatus	Tuz Lake, Karataş, Adana	36°42'20.8"N 35°03'08.2"E	3	OL739303
Kosswigichthys asquamatus	Behramaz Stream, Maden, Elazığ	38°28'27.5"N 39°33'26.6"E	5	OL739304- OL739307
Anatolichthys anatoliae	İnsuyu Stream, Cihanbeyli, Konya	38°43'22.2"N 32°43'02.7"E	6	OL739308- OL739311
Anatolichthys danfordii	Yay Lake, Develi, Kayseri	38°19'47.1"N 35°17'40.4"E	6	OL739313
Anatolichthys fontinalis	Salda Lake, Yeşilova, Burdur	37°31'35.0"N 29°40'29.8"E	4	OL739314
	Karaevli Spring, Burdur	37°34'46.7"N 30°24'02.5"E	2	OL739314
Anatolichthys iconii	Eğirdir Lake, Isparta	37°50'48.5"N 30°52'14.9"E	2	OL739315
	Yeşilyurt Village, Sütçüler, Isparta	37°31'57.5"N 30°51'53.0"E	2	OL739315
Anatolichthys irregularis	Aksu Stream, Kaklık, Denizli	37°50'08.3"N 29°26'02.6"E	2	OL739316
Anatolichthys maeandricus	Su çıkan Stream, Dinar, Afyon	38°04'54.7"N 30°09'35.3"E	3	OL739317
Anatolichthys marassantensis	Hirfanlı Dam, Kırşehir	39°01'59.5"N 33°59'24.6"E	3	OL739318
	Tuz Lake, Şerflikoçhisar, Ankara	38°54'11.8"N 33°24'58.6"E	4	OL739318
Anatolichthys meridionalis	Çataloluk Stream, Söğüt, Burdur	37°01'31.1"N 29°50'12.5"E	3	OL739319-OL739321
	Gavurçay Stream, Elmalı, Antalya	36°38'21.8"N 29°45'13.6"E	4	OL739322-OL739323
	Akçay Stream, Elmalı, Antalya	36°37'53.2"N 29°49'42.5"E	6	OL739322, OL739323, OL739326
	Aslanlı Stream, Gölhisar, Burdur	37°09'15.9"N 29°34'34.5"E	2	OL739327
Anatolichthys sureyanus	Eren Stream, Burdur Lake, Burdur	37°37'45.5"N 30°04'45.6"E	9	OL739328-OL739329
Anatolichthys transgrediens	Acı Lake, Başmakçı, Afyon	37°49'46.7"N 29°53'33.9"E	2	OL739330-OL739331
Anatolichthys villwocki	Özyurt, Polatlı, Ankara	39°12'38.5"N 32°04'12.8"E	3	OL739332
	Seydi Stream, Çifteler, Eskişehir	39°24'46.2"N 31°07'43.3"E	9	OL739333-OL739335
	Beşgöz Pond, Sarayönü, Konya	38°16'23.9"N 32°20'45.9"E	1	OL739333
Anatolichthys saldae	Salda Lake, Yeşilova, Burdur	37°31'35.0"N 29°40'29.8"E	4	OL739336-OL739339
Paraphanius alexandri	Çöçelli, Pazarcık, Kahramanmaraş	37°16'23.5"N 37°06'50.6"E	2	OL739340
Paraphanius orontis	Asi River, Samandağ, Hatay	36°04'57.1"N 35°57'07.8"E	2	OL739341
Paraphanius boulengeri	Gölbaşı Lake, Adıyaman	37°48'00.6"N 37°38'40.0"E	4	OL739342
Paraphanius similis	Bağlı Village, Aksaray	38°16'29.9"N 34°03'34.4"E	2	OL739343
Paraphanius mentoides	Nemrut Lake, Tatvan, Bitlis	38°37'07.6"N 42°12'39.1"E	5	OL739344
	Kırkgöz Lake, Döşemealtı, Antalya	37°04'32.0"N 30°34'14.4"E	2	OL739344
	Düden Stream, Kepez, Antalya	36°57'11.9"N 30°44'26.0"E	5	OL739345-OL739346
Total			**107**	**44**

The mtDNA *cytb* gene of 107 individuals belonging to the Aphaniidae family was amplified and sequenced 1065 nucleotide without insertion, deletion, gap and stop codon. The *cytb* sequences were deposited in GenBank under the accession numbers OL739303-OL739346. The average nucleotide composition for 107 *cytb* sequences is 30.8% T, 28.8% C, 25.0% A and 15.5% G. For Aphaniidae species, 683 (64.1%) of the mtDNA *cytb* nucleotide sequences were conserved, 382 (35.9%) variable and 382 (35.9%) parsimonic informative. For polymorphic nucleotide positions, 92 transitions and 39 transversions were determined, and the ratio of transition (Ti) to transversion (Tv) was calculated as Ti/Tv=2.36.

A total of 44 haplotypes of 19 Aphaniidae species distributed in Anatolia were determined. Most of the species are represented by only 1 haplotype, while *Anatolichthys* meridionalis is represented by 9 haplotypes.

The number of haplotypes, the haplotype diversity and nucleotide diversity values are shown in Table 2.

The haplotype diversity of *Anatolichthys saldae* and *Anatolichthys transgrediens* populations were the highest (Hd = 1.000), compared with the lowest haplotype diversity of *Aphanius fasciatus*, *Anatolichthys fontinalis*, *Anatolichthys iconii*, *Anatolichthys irregularis*, *Anatolichthys maeandricus*, *Anatolichthys marassantensis*, *Paraphanius alexandri*, *Paraphanius boulengeri*, *Paraphanius orontis* and *Paraphanius similis* (Hd = 0.0000). The nucleotide diversity of *Anatolichthys meridionalis* was the highest (Pi = 0.0086), compared with the lowest nucleotide diversity of *Aphanius fasciatus*, *Anatolichthys fontinalis*, *Anatolichthys iconii*, *Anatolichthys irregularis*, *Anatolichthys maeandricus*, *Anatolichthys marassantensis*, *Paraphanius alexandri*, *Paraphanius boulengeri*, *Paraphanius orontis* and *Paraphanius similis* (Pi = 0.0000) (Table 2).

Table 2. Sample size (N), number of haplotypes (HN), haplotype diversity (Hd) and nucleotide diversity (Pi) for Aphaniidae species.

Species	N	HN	Hd	Pi
Aphanius fasciatus	3	1	0.0000	0.0000
Kosswigichthys asquamatus	5	4	0.9000	0.0030
Anatolichthys anatoliae	6	4	0.8000	0.0020
Anatolichthys danfordii	6	2	0.5333	0.0005
Anatolichthys fontinalis	6	1	0.0000	0.0000
Anatolichthys iconii	4	1	0.0000	0.0000
Anatolichthys irregularis	2	1	0.0000	0.0000
Anatolichthys maeandricus	3	1	0.0000	0.0000
Anatolichthys marassantensis	7	1	0.0000	0.0000
Anatolichthys meridionalis	15	9	0.8857	0.0086
Anatolichthys sureyanus	9	2	0.5000	0.0005
Anatolichthys transgrediens	2	2	1.0000	0.0009
Anatolichthys villwocki	13	4	0.7949	0.0020
Anatolichthys saldae	4	4	1.0000	0.0020
Paraphanius alexandri	2	1	0.0000	0.0000
Paraphanius orontis	2	1	0.0000	0.0000
Paraphanius boulengeri	4	1	0.0000	0.0000
Paraphanius similis	2	1	0.0000	0.0000
Paraphanius mentoides	12	3	0.6212	0.0013
TOTAL	**107**	**44**		

The intergeneric distances among Aphaniidae genera ranged from 23.1% (between *Aphanius* and *Paraphanius*) and 16.6% (between *Aphanius* and *Anatolichthys*) (Table 3). The intrageneric distance within Aphaniidae genera is 0% (*Aphanius*) and 8.9% (*Anatolichthys*) (Table 3).

Table 3. Average intergeneric and intrageneric distance for Aphaniidae family. The parts marked in gray indicate average genetic distances within genera.

	Genera	1	2	3	4
1	*Aphanius*	0.000			
2	*Kosswigichthys*	0.183	0.003		
3	*Anatolichthys*	0.166	0.169	0.089	
4	*Paraphanius*	0.231	0.219	0.222	0.041

For *cytb*, intraspecies and interspecies genetic distance values are given in Table 4. The interspecific genetic distances among Aphaniidae species ranged from 0.1% (between *Anatolichthys fontinalis* and *Anatolichthys sureyanus*) and 24.52% (between *A. villwocki* and *P. mentoides*) (Table 4). On the other hand, the intraspecific genetic distances within Aphaniidae species ranged from 0% (*Aphanius fasciatus*, *Anatolichthys marassantensis*, *Anatolichthys iconii*, *Anatolichthys sureyanus*, *Anatolichthys fontinalis*, *Anatolichthys maeandricus*, *Anatolichthys irregularis*, *Paraphanius boulengeri*, *Paraphanius similis*, *Paraphanius mentoides*, *Paraphanius orontis*, *Paraphanius alexandri* and) and 0.9% (*Anatolichthys meridionalis*) Table 4.

Phylogenetic analyses were performed with distance-based (NJ) and character-based (ML and MP) methods based on the mtDNA *cytb* gene. The tree topologies produced by the three methods were generally compatible with each other. Anatolian Aphaniids, *Anatolichthys*, *Paraphanius*, *Aphanius*, *and Kosswigichthys* genera, constructed four lineages in tree topology. The phylogenetic trees recovered by NJ, MP and ML methods yielded identical topologies with high bootstrap supports (51-100% for NJ, MP, and ML) (Figure 1).

Table 4. Intraspecies and interspecies average genetic distance values of Aphaniidae family. The parts marked in gray indicate average genetic distances within species.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	*A. fasciatus*	0.000																		
2	*K. asquamatus*	0.183	0.003																	
3	*A. anatoliae*	0.169	0.165	0.002																
4	*A. marassantensis*	0.156	0.165	0.105	0.000															
5	*A. iconii*	0.173	0.155	0.089	0.114	0.000														
6	*A. villwocki*	0.165	0.166	0.107	0.113	0.109	0.002													
7	*A. transgrediens*	0.180	0.179	0.104	0.121	0.062	0.115	0.001												
8	*A. meridionalis*	0.152	0.165	0.097	0.100	0.082	0.102	0.105	0.009											
9	*A. fontinalis*	0.174	0.176	0.105	0.123	0.065	0.116	0.077	0.093	0.000										
10	*A. maeandricus*	0.180	0.177	0.092	0.102	0.101	0.114	0.117	0.103	0.115	0.000									
11	*A. danfordii*	0.161	0.170	0.102	0.077	0.107	0.109	0.121	0.101	0.105	0.109	0.001								
12	*A. irregularis*	0.183	0.178	0.093	0.102	0.104	0.114	0.119	0.106	0.118	0.006	0.109	0.000							
13	*A. sureyanus*	0.173	0.174	0.104	0.122	0.063	0.115	0.075	0.092	0.001	0.116	0.106	0.119	0.000						
14	*A. saldae*	0.172	0.172	0.107	0.127	0.061	0.121	0.070	0.093	0.031	0.121	0.110	0.123	0.030	0.002					
15	*P. boulengeri*	0.227	0.220	0.206	0.220	0.221	0.229	0.221	0.219	0.222	0.229	0.214	0.229	0.224	0.222	0.000				
16	*P. similis*	0.227	0.219	0.203	0.224	0.214	0.230	0.221	0.214	0.220	0.232	0.216	0.235	0.222	0.221	0.028	0.000			
17	*P. mentoides*	0.237	0.223	0.212	0.218	0.223	0.245	0.217	0.225	0.222	0.222	0.217	0.228	0.223	0.217	0.065	0.071	0.001		
18	*P. orontis*	0.215	0.199	0.193	0.207	0.206	0.218	0.199	0.208	0.213	0.212	0.215	0.212	0.214	0.208	0.059	0.076	0.064	0.000	
19	*P. alexandri*	0.222	0.216	0.206	0.214	0.228	0.228	0.221	0.216	0.221	0.230	0.217	0.230	0.222	0.224	0.019	0.032	0.065	0.062	0.000

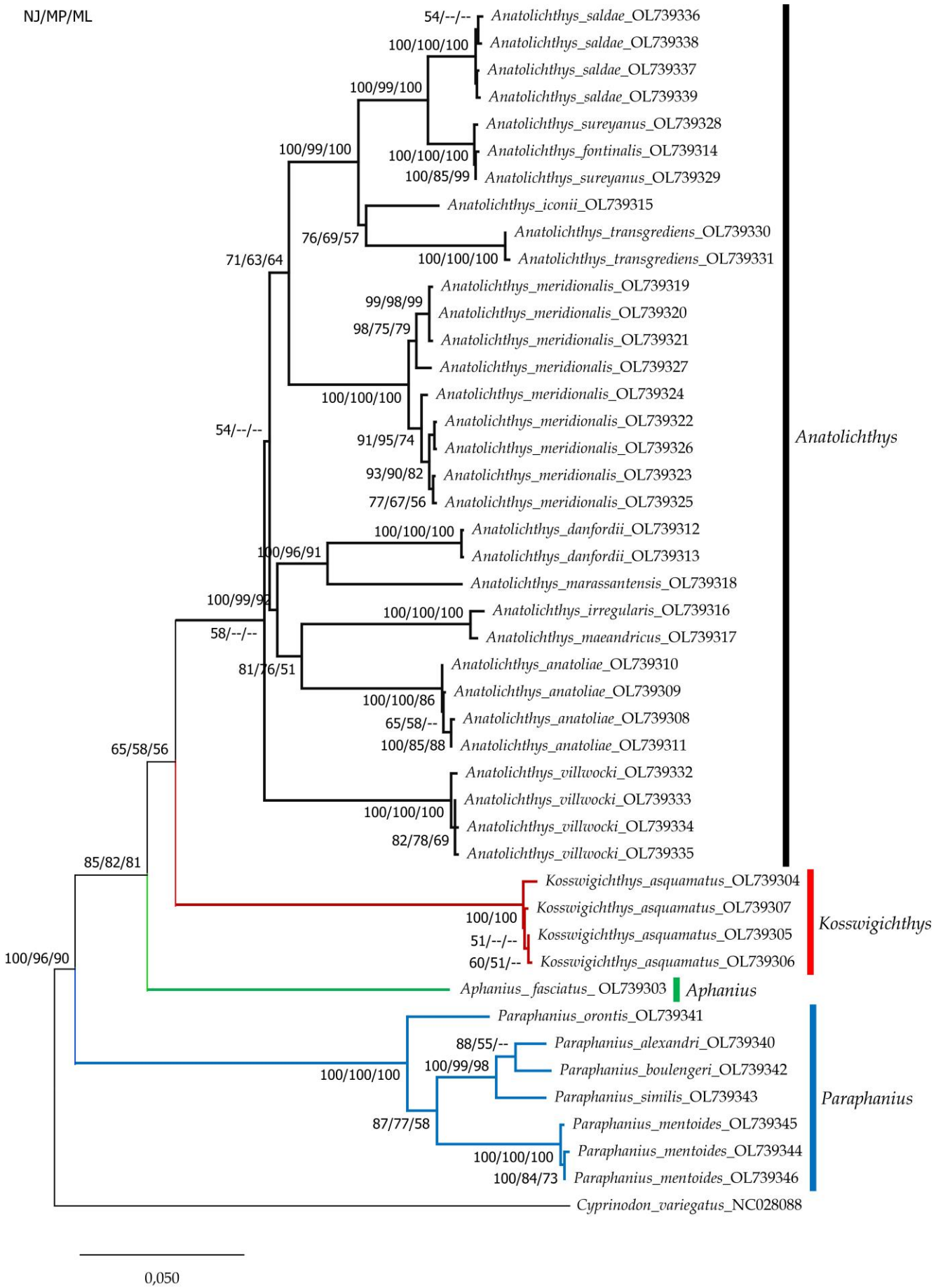

Figure 1. NJ phylogenetic tree generated based on the mitochondrial *cytb* gene. NJ, MP, and ML methods yielded the same topologies, and therefore only the NJ tree is shown. The bootstrap values are indicated on nodes (NJ/MP/ML).

DISCUSSION AND CONCLUSION

The sample size of our study was limited in order to protect populations, as most of the Aphaniids are threatened with extinction according to the IUCN (The International Union for Conservation of Nature) Red List. Therefore, a sufficient number of samples could not be collected from some locations. Aphaniids are represented in Anatolia by four genera (*Aphanius*, *Kosswigichthys*, *Anatolichthys* and *Paraphanius*). Nineteen out of 21 valid species distributed in Anatolia according to the literature were used in this study. *A. splendens*, which was recorded only from Lake Gölcük, could not be included in our study because it is probably extinct (Freyhof & Yogurtcuoglu, 2020). *Aphanius almiriensis* could not be sampled during fieldwork.

The partial *cytb* gene (1065 bp) of mitochondrial DNA was sequenced for 107 specimens of 19 aphaniid species distributed in Anatolia. Forty-four *cytb* haplotypes, all species-specific, were obtained from this dataset.

A pattern in which nucleotide transitions are favored several-fold over transversions is common in molecular evolution (Stoltzfus & Norris, 2015). This theory suggests that selection on proteins plays at least a minor role in the observed bias. Therefore, the Ti/Tv ratio, which is often greater than 0.5, has been used as an important parameter such as phylogenetic tree construction and estimation of divergence (Wang et al., 2015). It is also a way to measure the degree of multiple base substitution that has occurred since the common ancestor of the two sequences (Kocher & Stepien, 1997). Ti/Tv ratio (2.36), which was calculated for the present dataset, is consistent with studies (e.g., Jacquier, et al., 2013; Bloom, 2014; Firnberg, et al., 2014) that provide direct evidence on the relative conservativeness of transitions and transversions that change amino acids and therefore, Anatolian Aphaniid species indicate a relatively low level of genetic variation. High haplotype diversity and low nucleotide diversity determined for Aphaniid species (*K. asquamatus*, *A. anatoliae*, *A. danfordii*, *A. meridionalis*, *A. saldae*, *A. sureyanus*, *A. transgrediens*, *A. villwocki* and *P. mentoides*) distributed in Central Anatolia (Table 2) can be explained by the fact that populations of species distributed in the Central Anatolian plateau including the Göller district, which is known to occur more recently than in other geographical regions, may have exhibited a rapid population expansion and mutation accumulation following the genetic bottleneck. In fact, the evolutionary history of the Anatolian *Pseudophoxinus* (Hrbek et al., 2004) and *Aphanius anatoliae* (Hrbek et al., 2002) species complexes is nearly identical. It is thought that the low haplotype and nucleotide diversity values determined for the other Anatolian aphaniid species may be due to the insufficient number of samples.

It was found that the genetic distance values (Table 4) among Aphaniid species were compatible with both the genetic relationship model suggested by Bardakçı et al., (2004) based on RAPD analysis, and the genetic distance values obtained from the analysis of the *Aphanius* species complex based on 2 ribosomal RNA gene sequences by Hrbek et al., (2002). The genetic distances between *Anatolichthys fontinalis* - *Anatolichthys sureyanus* (0.13%, Table 4) and *Anatolichthys maeandricus* - *Anatolichthys irregularis* (0.57%, Table 4) are very low compared to other species, indicating that these species diverged recently.

Phylogenetic trees, which were constructed using three different algorithms (ML, MP, and NJ), exhibited largely consistent phylogenies for the Aphaniid species. These topologies showed that Anatolian Aphaniid species included four lineages corresponding to genera (*Anatolichthys*, *Paraphanius*, *Aphanius*, and *Kosswigichthys*), with high bootstrap values (Figure 1) and that is largely consistent with known phylogenetic relationships (Hrbek et al., 2002; Hrbek & Meyer, 2003; Esmaeili et al., 2020; Freyhof & Yogurtçuoğlu, 2020). In addition, the mean genetic distance between these four genera varies between 16.6% (*Anatolichthys*) and 23.1% (*Paraphanius*), supporting phylogenetic lineages that are monophyletic. The genera *Aphanius* and *Kosswigichthys* could not be tested because they are represented by a single taxon. *A. fontinalis*, *A. sureyanus*, *A. maeandricus*, and *A. irregularis* are closely related in the phylogenetic tree, consistent with genetic distance results (Figure 1).

While the tooth-carp specimens of Konya Beşgöz pond differed from all other tooth-carp species in the nearby geographically located lakes region, they clustered together with the specimens from the type locality of *A. villwockii* described by Hrbek and Wildekamp (2003) in the Sakarya basin (Figure 1). The fact that Freyhof (2014) previously reported that *A. villwocki* has distributed in Sakarya River and Ilgın Lake also supports this close relationship. Also, Geiger et al. (2014) reported a very recent biogeographic connection between the Sakarya River and Ilgın Lake basins, and stated that *Alburnus nasreddini* and *Squalius recurvirostris* in Ilgın Lake are more closely related to *S. pursakensis* and *A. escherichii* in Sakarya River. In addition, Aksu and Bektaş (2019) determined that *G. fahrettini*, which was identified from Lake Ilgın, is expected to be genetically close to other species in the Göller district (which is geographically closer to Lake Ilgın), and is closely related to it. *G. sakaryaensis* from the Sakarya River basin (geographically further away from Ilgın Lake). This phenomenon can be explained by the possibility of connecting the Upper

Sakarya Basin to the Ilgın Lake basin by a large freshwater paleo lake that continued until the upper Neogene period in Central Anatolia (Popov et al., 2004).

Turkey is a speciation center for the family Aphaniidae, as it has 19 species and a high rate of endemism. However, the species belonging to this ecologically important family have become unable to survive due to various factors. Anatolian aphaniids are threatend by climate change (1), food pollution by chemical pesticides and fertilizer waste in wetlands (2), destruction of reeds and wetlands, which are their habitat for irrigation projects (3). In addition, invasive species such as the mosquito fish *Gambusia holbrooki*, introduced in Turkey as part of biological control against mosquitoes, have a bit of competition, predation, and aggression pressure on Anatolian Aphaniids. *Aphanius splendens* is already extinct. As a result, most aphaniid species are listed as threatened on the IUCN Red List. Because variable environmental conditions can influence species dispersal capacity and population structure (Schönhuth et al., 2003; Whitehead, 2009), the determination of species diversity, phylogenetic relationships, and distribution areas are necessary for the development of in situ conservation strategies.

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