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ARAŞTIRMA MAKALESİ
RESEARCH PAPER

Molecular Phylogeny and Historical Biogeography of *Petroleuciscus* (Teleostei: Leuciscidae) Species in Turkey

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Abstract: Palaearctic leuciscid genus *Petroleuciscus* is a poorly known genus and molecular phylogeny and historical biogeography is not known well. In this study, phylogenetic relationships of *Petroleuciscus* species in Turkey were investigated by analyzing mitochondrial cytochrome b (cyt b) gene sequences (1140 bp). For this purpose, cyt b gene of 84 samples from 13 populations of the species were sequenced. Twenty-one haplotypes were identified and overall average haplotype diversity (Hd) was calculated as 0.921±0.011. The result of phylogenetic analysis of *Petroleuciscus* species shows the presence of two clades and four haplogroups. First clade includes *Petroleuciscus smyrneaus* and *P. ninae* from the Western Anatolia. Second clade indicated that *P. borysthenicus* consists of the Black Sea and the Marmara Sea drainages and northern part of the Aegean Sea drainages. Haplotype network analysis, phylogenetic and phylogeographic inference show that *Petroleuciscus* genus was monophyletic in Turkey, and its species scattered in the Miocene era.

Keywords: cyt b, genetic diversity, leuciscids, mtDNA, phylogeography.

Türkiye'deki *Petroleuciscus* (Teleostei: Leuciscidae) Türlerinin Moleküler Filogenisi ve Tarihsel Biyocoğrafyası

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Öz: Palaearktik leuciscid cinsi *Petroleuciscus*, az bilinen bir cins olup moleküler filogenisi ve tarihi biyocoğrafyası iyi bilinmemektedir. Bu çalışmada, Türkiye'deki *Petroleuciscus* türlerinin filogenetik ilişkileri mitokondriyal sitokrom b (cyt b) (1140 bp) geni kullanılarak araştırılmıştır. Bu amaçla, 13 populasyondan 84 örneğin cyt b geni dizilenmiştir. Genetik analizler sonucunda 21 haplotip tanımlanmış ve haplotip çeşitliliği Hd: 0,921±0,011 olarak hesaplanmıştır. *Petroleuciscus* türlerinin filogenetik analizinin sonucunda ise, iki klad ve dört haplogrup varlığı ortaya çıkmaktadır. Birinci klad, Batı Anadolu'dan *Petroleuciscus smyrneaus* ve *P. ninae*' den oluşmaktadır. İkinci klad ise, *P. borysthenicus*'un Karadeniz ve Marmara Denizi havzasından ve Ege Denizi'nin kuzey sularından oluştuğunu ortaya çıkarmaktadır. Haplotip ağ analizi, filogenetik ve filocoğrafik çıkarım, Türkiye'deki *Petroleuciscus* cinsinin monofiletik olduğunu ve türlerinin Miyosen çağında dağıldığını göstermektedir.

Anahtar kelimeler: cyt b, filocoğrafya, genetik çeşitlilik, leuciscidler, mtDNA .

INTRODUCTION

The members of genus *Petroleuciscus* are widely distributed in European rivers, having highest diversity particularly in the Balkan area and the southern European region. Their adults exist in lowland rivers, lakes, limans, deltas, lower parts of montane rivers, and backwaters. They can tolerate low oxygen concentrations and slightly brackish water. Nowadays, some populations are exposed to sharp decline due to river beds channelization and floodplains drainage. Palaearctic leuciscid genus *Petroleuciscus* was described by Bogutskaya, (2002). This genus is phenotypically and genetically distinct from sister group *Squalius* (Perea et al. 2010; Schönhuth et al. 2018). Up to now from its identification, *Petroleuciscus* genus is exposed to some taxonomic revisions, which are summarized by Freyhof et al., (2018). Now the genus *Petroleuciscus* consists of six species as *P. borysthenicus* (Kessler, 1859); *P. smyrnaeus* (Boulenger, 1896), *P. ninae* Turan, Kalaycı, Kaya, Bektaş & Küçük, 2018, *P. squaliusculus* (Kessler, 1872), *P. ulanus* (Günther, 1899) and *P. aphipsi* Aleksandrov, 1927.

Petroleuciscus borysthenicus is distributed in Eurasia: Eastern, northern, and western, Black Sea and Azov Sea basins, from eastward in Europe to northwestern Turkey. *P. smyrnaeus* is distributed in Asia and Europe: Lesbos Island in Greece and Aegean drainages of southwestern Turkey, Gediz River drainages (the northern Aegean Sea basin). *P. ninae* is distributed in Büyük Menderes drainage (the southern Aegean Sea basins). *P. squaliusculus* is distributed in Syr Darya drainage, Kyrgyzstan, Kazakhstan, and Tajikistan. *P. ulanus* is distributed from Orumeyeh Lake in Iran. Lastly, *Petroleuciscus aphipsi* is found in Kuban drainage, Russia (Bogutskaya, 1996; Froese & Pauly 2022; Turan et al., 2018).

Main factor of Leuciscinae distribution in the Europe is Pleistocene glaciations and Danube basin, which is glacial refuge that is origin of some leuciscine taxa after colonization. Additionally, in the formation of freshwater ichthyofauna, Black sea rivers also could have behaved as glacial refuge (Kotlík et al. 2004; Perea et al. 2010). Sanjur et al., (2003) pointed out that *Squalius borysthenicus* and *Squalius smyrnaeus* are paratethyan origin and distributed around the ancient Paratethys Sea cohorent to Doadrio and Carmona, (1998) and Zardoya et al., (1999).

Some phylogenetic and taxonomic studies were carried on the leuciscids in Turkey. Phylogenetic relationships of 190 European leuciscid species were investigated based on cytochrome b (cyt b) sequences and dispersal of species base Messinian period (Levy et al. 2009). Phylogenetic relationships between *Alburnoides* species revealed by Bektas et al. (2019) indicate that Turkish populations dispersed tectonic faulting and uplift Miocene aged as well as climatic variation and change in sea-level during late Miocene-middle Pleistocene. Namely, cyt b gene has been proven as an useful DNA markers for studying the systematics and phylogenetic of Leuciscidae and its congeners (Aksu & Bektaş 2019; Bektas et al. 2017; Durand et al. 2000; Durand, et al. 2002; Bektas et al. 2017; Durand et al. 2000; Durand, et al. 2002; Kuyumcu et al. 2021; Levin et al. 2012; Tsigenopoulos et al. 2002; Zardoya & Doadrio 1999).

Some *Petroleuciscus* samples were studied by Durand et al. (2000), Perea et al. (2010), Geiger et al. (2014), Freyhof et al. (2018) and Turan et al. (2018), but only one of these studies (Turan et al., 2018) is included limited COI barcoding data for all *Petroleuciscus* species distributed in Turkey for genetic assignment of species. Hence, as stated by Perea et al. (2010) *Petroleuciscus* genus is a still poorly known genus and real taxonomy is not known well.

Considering that three of the six species of the genus *Petroleuciscus* are distributed in Turkey, to reveal the phylogenetic relationships of species distributed in Turkey is very important to determine systematic position and phylogeographic history of the *Petroleuciscus*. Unfortunately, previous studies were not adequate to reveal the phylogenetic, phylogeographic relationships and specification of *Petroleuciscus* distributed in Turkey. For this reason, in this study, we aimed to investigate phylogenetic relationships and delineate phylogeographic history of *Petroleuciscus* genus in Turkey.

MATERIAL AND METHOD

***Sample collection, DNA Extraction and Sequencing*:** Three *Petroleuciscus* species of Turkey were collected from 13 sampling sites and 84 specimens from drainages of the Black Sea, the Aegean Sea, and the Marmara Sea basin (Table 1, Figure 1). Note that some samples were also used Turan et. al., (2018). The care of experimental animals was consistent with Republic of Turkey animal welfare laws, and guidelines. Fishes were collected, surgical procedures were only performed for excision of fin clips after anaesthesia by using MS222, and then the fin clips preserved in 70% ethanol for DNA extraction. Total DNA was extracted from fin clips via Qiacube automated DNA/RNA purification system using Qiagen DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany). The quality and quantity of DNA were checked on both the NanoDrop 2000/c spectrophotometer (Thermo Scientific, Rockford, IL, USA) and 0.8% agarose gel electrophoresis. Mitochondrial cytochrome b (cyt b) gene (1141 bp) was amplified by primer set B-cytbF (5'-GAAGAACCACCGTTGTWVTTCAAC-3') and the B-cytbR (5'- CGGATTACAAGACCGATGC-3') following the protocol and conditions described in Kalaycı, (2022). The PCR products were run 1% agarose gel electrophoresis and visualized under UV Quantum–Capt ST4 system (Vilber Lourmat, France). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions and bidirectional sequencing of PCR products was performed with an ABI PRISM 3730XL Genetic Analyser at Macrogen Europe Inc. (Amsterdam, Netherlands).

Table 1. Locality no, species, locality, and coordinates of *Petroleuciscus* species in Turkey.

Locality no	Species, Locality	Coordinates
	P. borysthenicus	
1	İnece stream, Nothern Aegean sea slope, Edirne	41°41'34.0"N, 27°04'59.0"E
2	Bahçeköy stream, Black sea drainage, Kırklareli	41°34'09.0"N, 28°04'46.0"E
3	Binkılıç stream, Black sea drainage, İstanbul	41°22'48.3"N, 28°17'46.0"E
4	Çamaşır stream, Marmara sea slope (Northern), İstanbul	41°09'48.6"N, 28°27'33.3"E
5	Sakan stream, Marmara sea slope (Southern), Balıkesir	40°03'00.0"N, 27°57'46.0"E
6	Balıklı stream, Marmara sea slope (Southern), Çanakkale	39°57'42.2"N, 27°12'53.9"E
	P. smyrnaeus	
7	Kocaçayı stream, Gediz drainage, İzmir	38°48'36.5"N, 27°01'48.3"E
8	Bakırçay stream, Gediz drainage, İzmir	39°07'57.0"N, 27°22'18.0"E
9	Gördes stream, Gediz drainage, Manisa	38°44'34.4"N, 27°55'47.0"E
10	Gölcük lake, Gediz drainage, İzmir	38°18'39.5"N, 28°01'52.2"E
11	Gediz River, Uşak	38°46'37.2"N, 29°12'40.4"E
	P. ninae	
12	Yenişehir pond, Küçük Menderes drainage, İzmir	38°04'30.0"N, 27°55'37.2"E
13	Akçay stream, Büyük Menderes drainage, Aydın	37°45'34.0"N, 28°20'07.0"E

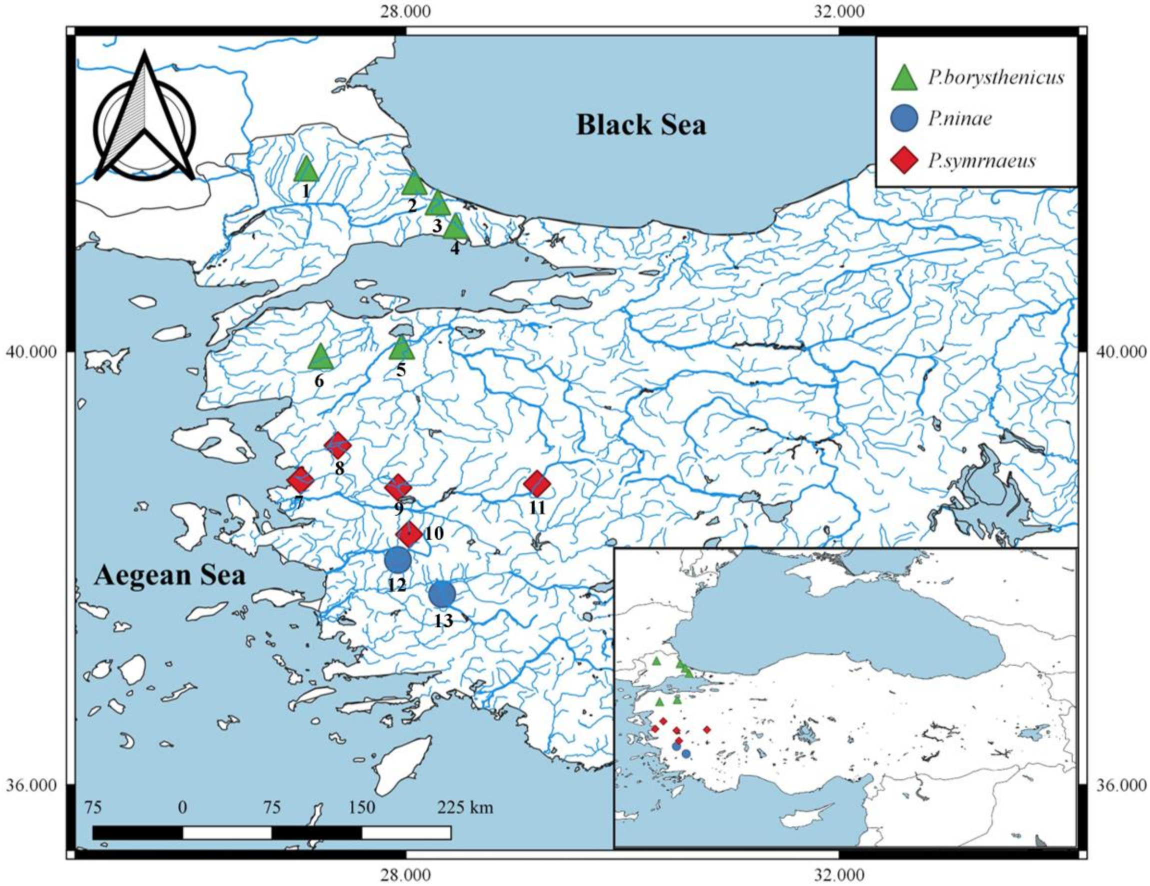

Figure 1. Map showing sampling area and stations.

Genetic Structure and Phylogenetic Analysis: The nucleotide sequences of cyt b gene was aligned using Clustal W algoritm (Thompson et al., 1994) implemented in Bioedit v7.2.5 (Hall, 1999) software. Haplotype number (H), haplotype diversity (Hd), and nucleotide diversity (π) were computed for each species using DnaSP version 6.12.03 (Rozas et al., 2017) program. Sequences of haplotypes were submitted to the NCBI GenBank with accession numbers MN877324- MN877344. Nucleotide frequencies, variable and polymorphic nucleotides, and transition/transversion rate were calculated using MEGA X (Kumar et al. 2018). To calculate genetic variation among groups and within the groups analysis of molecular variance (AMOVA) was conducted using the Arlequin v3.5.1.2 (Excoffier & Lischer, 2010) software. According to Bayesian information criterion (BIC) and Akaike information criterion (AIC) implemented in jModeltest v. 0.0.1 (Posada, 2008), the TrN+I model: -ln= 2427.5947 (Tamura K & Nei, 1993) was used as best-fit nucleotide substitution model for phylogenetic analysis. Median-joining (MJ) algorithm (Bandelt et al., 1999) implemented in Network 5.0.0.1 software (www.fluxusengineering.com) was used to construct the haplotype network. Phylogenetic relationships among haplotypes and species were estimated using maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI) analysis using MEGA X (Kumar et al. 2018), PAUP 4.0b10 (Swofford, 2003) and MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) softwares, respectively. At the BI analysis, Metropolis-coupled Markov chain Monte Carlo (MCMC) algorithm was used for one million generations and sampled every 1000 generations. Sampled in each MCMC run, initial 25% of saved trees discarded as burn-in. *Ladigesocypris irideus* (HM560096 for cyt b) was selected as outgroup taxa for all phylogenetic analyses. Pairwise genetic distance estimation among the species was calculated by MEGA X (Kumar et al., 2018) software using the Kimura 2-parameter substitution model (Kimura, 1980).

The Bayesian MCMC coalescent method implemented in BEAST v1.8.2 (Drummond et al., 2012) was used to divergence time estimation. The molecular clock calibration was used 0.4% per lineage per million years based on divergence rate of cyt b gene in Leuciscinae concluded by Perea et al. (2010) and this rate was also used by (Buj et al., 2019). Branch rates were determined from an uncorrelated lognormal distribution and a Yule speciation prior with random starting tree. Nucleotide substitution model, TrN was applied with Gamma site heterogeneity model. The MCMC steps number was three million with parameters logged every 1000 generations. The remaining parameters in the software were chosen as default. Produced trees were summarized by TreeAnnotator v.1.8.2 to obtain a maximum clade credibility tree with 25% burn-in. The tree displayed with FigTree v. 1.4.4. node labels height_95% HPD and height median. Effective sample size and convergence of parameters was evaluated using Tracer v.1.6. The effective sample sizes for all parameters of interest were greater than 200.

RESULTS

Genetic Diversity and Species Divergence: Nucleotide sequences of the cyt b gene region (1140 bp) were examined in 84 specimens belonging to three species of *Petroleuciscus* in Western Anatolia and Thrace region (Table 2). The overall average nucleotide frequencies were determined as 26.09% A, 28.60% T, 28.43% C, and 16.88% G. The transition/transversion rate k1 = 42.723 (purines), k2 = 14.786 (pyrimidines) and the overall transition/transversion bias were calculated as R=12.584. Haplotype number was determined as 21; eight from *P. borysthenicus* (N=31), ten from *P. smyrnaeus* (N=32) and three from *P. ninae* (N=21) (Table 2).

Table 2. Frequency, distribution, and Genbank accession numbers of Cyt b and haplotypes of *Petroleuciscus* in Turkey (the locality number is in parentheses).

		P. borysthenicus						***P. smyrnaeus***					***P. ninae***	
Haplotypes	**Genbank Accession Number**	**İnece S. (1)**	**Sakan S. (5)**	**Balıklı S. (6)**	**Çamaşır S. (4)**	**Bahçeköy S. (2)**	**Binkılıç S. (3)**	**Gediz R. (11)**	**Gördes S. (9)**	**Kocaçayı S. (7)**	**Bakırçay S. (8)**	**Gölcük L. (10)**	**Akçay S. (13)**	**Yenişehir P. (12)**
H1	MN877324	6												
H2	MN877325	1												
H3	MN877326		4											
H4	MN877327		4	2										
H5	MN877328		1											
H6	MN877329			2										
H7	MN877330				3									
H8	MN877331					8	2							
H9	MN877332							6	5					
H10	MN877333								2					
H11	MN877334								1					
H12	MN877335									3	8			
H13	MN877336									1				
H14	MN877337									1				
H15	MN877338									1				
H16	MN877339										1			
H17	MN877340											2		
H18	MN877341											1		
H19	MN877342												2	5
H20	MN877343												11	1
H21	MN877344													2
		7	**9**	**4**	**3**	**8**	**2**	**6**	**8**	**6**	**9**	**3**	**13**	**8**

Totally, 77 variable sites were identified and 71 of them were polymorphic. Haplotype and nucleotide diversity are *P. borysthenicus*, *P. smyrnaeus* and *P. ninae* (Hd=0.837±0.040 and π=0.00584±0.00032), (Hd=0.774±0.052 and π=0.00273±0.00032), (Hd=0.581±0.075 and π=0.00569±0.00029), respectively. Overall haplotype and nucleotide diversity are Hd= 0.921±0.011 and π=0.02603±0.00126. The AMOVA suggested that almost all observed genetic variations occur among groups determined as 91.76% for three species. Haplotype network analysis contains 21 unique haplotypes and no haplotype shared between species, which are clearly separated species from each other by at least 9 mutational steps. The most common haplotype was H20, and it was shared by Akçay Stream and Yenişehir Pond populations belonging to *P. ninae* (Figure 2). Pairwise genetic distances between species were calculated as 0.011 (*P. ninae* and *P. smyrnaeus*), 0.054 (*P. borysthenicus* and *P. smyrnaeus*) and 0.049 (*P. borysthenicus* and *P. ninae*).

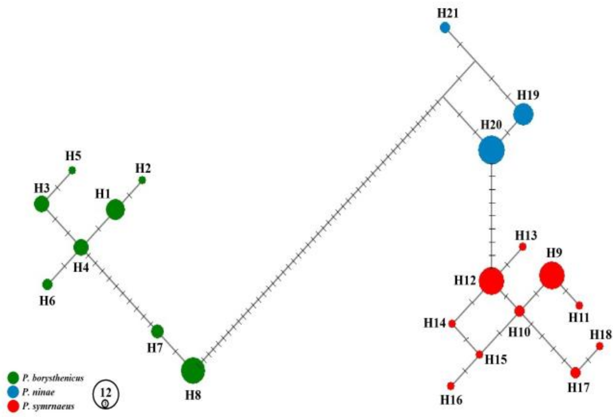

Figure 2. Median-joining network of the cytb haplotypes. Circle size corresponds to sample size; one bar indicates an additional mutational step. Each small lines represent one nucleotide difference.

Phylogenetic Relationships and Divergence Times: The phylogenetic inferences suggest that *Petroleuciscus* species in Turkey are clustered into two main clades. The first clade consists of *P. borysthenicus* while the other clade contains *P. smyrnaeus* and *P. ninae*. Maximum likelihood, maximum parsimony and Bayesian inference analyses of mitochondrial cyt b (Figure 3) gene resulted in trees congruent with each other and were supported by high bootstrap values (>75) for the distinction of species.

The divergence time between the two main clades indicates that the disperse of *Petroleuciscus* species in Turkey starts in 6.09 (10.8-3.36) mya Miocene (late Messinian stage) (Figure 4).

Figure 3. Maximum likelihood tree based on mitochondrial cytb gene sequences of *Petroleuciscus* species in Turkey. Maximum likelihood, Bayesian inference and Maximum Parsimony analyses resulted in congruent trees. Bootstrap and posterior probability values are shown above nodes on tree if 50 % or higher

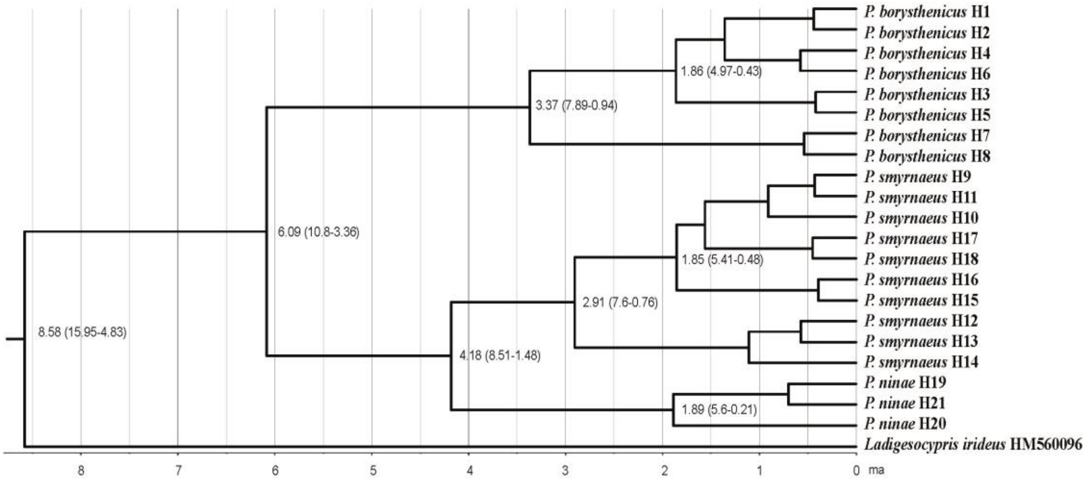

Figure 4. Divergence timescale for the *Petroleuciscus* species inferred under Bayesian uncorrelated relaxed clock method from mitochondrial cyt b genes. Numbers front the node represent divergence times in million years (Ma) and their HPD 95% credibility intervals.

DISCUSSION AND CONCLUSION

Phylogeny of two *Petroleuciscus* species distributed in Turkey (*P. borysthenicus* and *P. smyrneaus)* was studied by Perea et al. (2010) and it was indicated that the genus was not known sufficiently. In this study, the phylogeny constructed by ML, MP and BI approaches is based on mtDNA cyt b sequences of three *Petroleuciscus* species, which are clustered in two clades and four haplogroups with high bootstrap values and posterior probabilities. Our construction based on the mitochondrial cyt b gene lead to a remarkable topology, which places first lineage *P. borysthenicus,* and other lineage consists of *P. smyrneaus* and *P. ninae*. This result was also coherent by Turan et al., (2018) using a more conserved mt DNA gene, COI barcoding gene. First clade includes two haplogroups. The first one "H1, H2, H3, H4, H5, and H6" is found in Sakan, Balıklı stream (drainages of southern Marmara Sea) and İnece stream (a drainage of northern of Aegean Sea). The second one "H7 and H8" is found in Bahçeköy, Binkılıç streams (drainages of Black Sea and Thrace) and Çamaşır stream (drainage of northern of Marmara Sea). Second clade consists of two valid species which are identified before *P. smyrneaus* and *P. ninae*. Battalgil (1940) described *Leuciscus heterandrius* from the Lake İznik (southern Marmara Sea coast) but later it was synonymized with *L. borysthenicus* (Battalgil, 1942). Haplogroup H1-H6 in this phylogenetic tree may refer to *Petroleuciscus heterandrius*. The results of this study may suggest that four *Petroleuciscus* species may exist in Turkey based on the comparison of genetic distance for cyt b gene (0.011–0.054). Undoubtedly, validation studies should be done to clarify this inference, and especially morphological characters should be examined. AMOVA also supports this inference because of almost all observed genetic variations (>86 %) occur among haplogroups. In addition, the median joining network analyses of *Petroleuciscus* species indicates the presence of three haplogroups divided as north and south of Marmara Sea.

Bektas et al. (2017) and Hrbek et al. (2004) previously reported that, with a general opinion Anatolian freshwater fish species have low nucleotide diversity and high haplotype diversity and this implies a smaller founder population and the population is in the expansion period. Similarly, it was determined that the population structure of *Petroleuciscus* species in terms of haplotype and nucleotide diversity is in accordance with this general opinion.

Popov et al., (2004) refer that Central Europe and Anatolia were connecting from the late Oligocene to late Miocene (23-5.33 mya). Estimated dates of divergence time of *Petroleuciscus* from sister genus *Ladigesocypris* was estimated as 8.58 (15.95-4.83) mya and it might be dispersed from the Danube basin to Anatolia via river capture during this period. Similar to this result, (Levy et al., 2009) also calculated that divergence time of *Petroleuciscus* genus was about 13 mya. Divergence time between two main clades indicates that the divergence among the *Petroleuciscus* species starts in 6.09 (10.8-3.36) mya Miocene (late Messinian stage).

Divergence time between *P. smyrnaeus* and *P. ninae* was estimated as 4.18 (8.51-1.48) mya. Por (1989) indicate that a line drawn from west to east in the middle of Turkey can be considered as a major suture, and this lead to species differentials of *Petroleuciscus* between Büyük Menderes and Gediz River basin. *P. borysthenicus* clade divided into two haplogroups dated 1.86 mya (early Pleistocene). Durand et al. (2000) suggest that the most important component of Chub's evolutionary history is the Danube particularly during the Pleistocene glaciations since it was a refugium for the chub. Our molecular data confirm this influence speciation of *P. borysthenicus* and *P. heterandrius*, and it shows a reasonable geographical model.

Consequently, *Petroleuciscus* species in Turkey were analyzed based mt DNA cyt b gene and determined as monophyletic and differentiated from the sister group at Miocene era. Also, it is estimated that the divergence time of the two main clades which are the first clade including *P. borysthenicus*, and the second clade including *P. smyrnaeus, P. ninae* starts in Miocene (late Messinian stage). Speciation dynamics of *Petroleuciscus* species in Turkey have been determined and controlled by biogeographical processes after Miocene.

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