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RESEARCH PAPER

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DNA Barcoding of the Genus *Capoeta* (Actinopterygii: Cyprinidae) from Anatolia

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Abstract

The mitochondrial DNA cytochrome oxidase subunit I (COI) gene sequences were used as a DNA barcode to identify species of the *Capoeta* genus in Anatolia and to clarify their systematics. A 652-bp region of the mitochondrial cytochrome oxidase subunit I (COI) was evaluated on 253 individuals representing seventeen species of *Capoeta* and thirty-three haplotypes were totally identified. The COI barcoding efficiency test for Anatolian *Capoeta* species provided twelve congruent numbers of putative species for four delimitation methods (BCM, K2P, ABGD, and NJ). The mean congeneric genetic distance (4.01%) was approximately 14-fold higher than the mean of conspecific one (0.28%) and there was a clear-cut barcode gap (0.92 -1.26%) between maximum intraspecific and minimum nearest neighbour distance for Anatolian *Capoeta* species. The ML and BI phylogenies indicated a consensus tree topology containing three clades corresponding to the geographical origins: Anatolian-Iranian, Mesopotamian and Aralo-Caspian groups. The results of present study indicated that the COI gene could be a suitable DNA barcode marker for the most of scrapers species identification and delimitation with approximately 81% success rate.

Introduction

The rheophile species belonging to the genus *Capoeta* Cuvier & Valenciennes (1842) in the family Cyprinidae are distributed in a wide geographical region from East Europe to West Asia including Anatolia (Bănărescu, 1999; Levin *et al.,* 2012; Bektaş *et al.,* 2017). Up to now, thirty species of the *Capoeta* genus have been identified throughout Eurasia (Froese & Pauly, 2018), twenty-one of which are distributed in Anatolia (Turan, Kottelat, Kirankaya, & Engin, 2006a; Turan, Kottelat, Ekmekçi, & Imamoğlu, 2006b; Geldiay & Balık, 2007; Turan, Kottelat, & Ekmekçi, 2008; Turan, Küçük, Kaya, Güçlü, & Bektaş, 2017; Elp, Osmanoğlu, Kadak, & Turan, 2018). Out of twenty-one Anatolian *Capoeta* species, six are cross-border (*C. barroisi*, *C. capoeta*, *C. damascina*, *C. trutta*, *C. umbla* and *C. ekmekciae*), fifteen are endemic to Turkish freshwater fauna (*C. angorae*, *C. antalyensis*, *C. aydinensis*, *C. baliki*, *C. banarescui*, *C. bergamae*, *C. kosswigi, C. caelestis, C. erhani*, *C. mauricii*, *C. oguzelii*, *C. pestai*, *C. sieboldii*, *C. tinca* and *C. turani*). Recent morphological studies (Turan *et al.,* 2006a; Turan *et al.,* 2006b; Turan *et al.,* 2008; Turan *et al.,* 2017; Elp *et al.,* 2018) and molecular studies on the *Capoeta* genus (Turan, 2008; Bektaş, Çiftçi, Eroğlu, & Beldüz, 2011; Levin *et al.,* 2012: Geiger *et al.,* 2014; Alwan, Zareian, & Esmaeili, 2016; Ghanavi, Gonzalez, & Doadrio, 2016; Jouladeh-Roudbar, Eagderi, Ghanavi, & Dadrio 2017; Zareian, Esmaeili, Heidari, Khoshkholgh, & Mousavi-Saber, 2016) to resolve taxonomic uncertainties caused by phenotypic plasticity (Berg, 1949; Banarescu, 1999; Doadrio & Madeira, 2004) have led to an increase in the number of species indicating that the taxonomy of the *Capoeta* species group has not yet been fully resolved. Recently, Bektaş et al. (2017) has been

genetically defined Anatolian *Capoeta* species with a extensive molecular research using cyt *b* gene sequences. And they suggested that *C. kosswigi*, *C. turani*, *C. mauricii*, and *C. angorae* are probably to be synonyms of *C. umbla*, *C. erhani*, *C. pestai*, and *C. damascina*, respectively. Hebert, Cywinska, Ball, & Dewaard, (2003a) suggested that the COI-based DNA barcoding, which reveals species-specific DNA profiles, can prevent diagnostic and phylogenetic problems that may be caused by deficiencies in species identification keys and by their phenotypic flexibility. Using the barcode region of about 648-655 bp at the 5 'end of the cytochrome oxidase subunit I (COI) gene on the mitochondrial DNA, species can be distinguished by determining the difference between the largest intraspecific distance and the smallest interspecific distance, called the "barcoding gap" (Hebert, Ratnasingham, & Dewaard, 2003b; Meyer, & Paulay, 2005). DNA barcodes are now widely used to efficiently recognize known species (Hubert *et al.,* 2008; Keskin, Ağdamar, & Tarkan, 2012; Keskin, & Atar, 2013a; Keskin, & Atar, 2013b; Bhattacharya *et al.,* 2016), to detect undescribed or cryptic species (Ward, Holmes, Zemlak, & Smith, 2007; Ward, Holmes, & Yearsley, 2008), to determine taxonomy (Rasmussen, Morrissey, & Hebert, 2009; Levin *et al.,* 2016; Rossini *et al.,* 2016; Gordeevaa, & Shakhovskoi, 2017).

The aim of this study is to reveal species delimitation and interspecific relationships and to identify Anatolian *Capoeta* species that constitutes about 70% of *Capoeta* species in the world, using COI-based barcoding region.

Material and Methods

Specimen Sampling

The sampling study involved the collection of 253 individuals belonging to the *Capoeta* genus was carried out at geographical locations in the rivers and lakes systems of Turkey, including type localities (Table 1) during 2011-2012. The sampling protocol was approved by the Ethics Committee of the Recep Tayyip Erdogan University (Reference no. 210/37). All specimens were identified at species level by experienced taxonomists according to taxonomic keys from Berg (1949), Turan *et al.,* (2006a), Turan *et al.,* (2006b), Kottelat & Freyhof, 2007, Turan et al. (2008), Özuluğ & Freyhof (2008), Schöter, Özuluğ, & Freyhof, (2009), Küçük et al. (2009), Turan et al. (2017) and Elp et al. (2018). Approximately 100 mg of white muscle tissue from each specimen was preserved in 96% ethanol for genomic DNA extraction and transferred to the RTE University Genetic Laboratory of the Faculty of Fisheries for subsequent analysis.

DNA Extractions, PCR Amplification and Sequencing

Genomic DNA was extracted from alcohol-preserved caudal fin tissues of samples using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and automated on the QIAcube robot (QIAGEN, Valencia, CA). The obtained DNA were stored in -20°C for PCR applications. Quality and quantity of extracted DNA were estimated using 2000c Spectrophotometer (Thermo Scientific, USA). Approximately, 652 bp were amplified from the 5' region of the COI gene using universal fish barcoding primers: FishF1 (5-TCAACCAACCAC AAAGACATTGGCAC-3) and FishR1 (5-TAGACTTCTGGGTGGCCAAAGAATCA-3) (Ward, Zemlak, Innes, Last, & Hebert, 2005).

PCR reactions were carried out as 50 µL total volumes containing 10 µl of 5x reaction buffer, 3.5µl $MgCl_2$; 4 µl of dNTPs, 1 µl of *Taq* DNA polymerase; 26.5 µl ultrapure water; 1µl of each COI primer (FishF1 and FishR1) and 3µl of DNA template. PCR reactions were performed using a T100^{TM} PCR Gradient Thermal Cycler (Bio-Rad, Hercules, USA). The PCR thermal cycling conditions was: 95ºC for 3 min; 36 cycles of 95ºC for 45 s, 52ºC for 30 s, 72ºC for 45 s; final extension of 72ºC for 5 min; and hold at 4ºC. Amplification products were visualized on 1.2% agarose gel stained with ethidium bromide. The PCR products were run on 1.5% agarose gel stained with ethidium bromide (0.5 µg/mL), in TAE buffer, and were visualized under UV Quantum-Capt ST4 system (Vilber Lourmat, France). The concentrations of purified PCR products were estimated by NanoDrop 2000C UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). Amplicons were sequenced bi-directionally using sequencing primers FishF1 or FishR1 (Ward, Zemlak, Innes, Last, & Hebert, 2005) and the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.) on an ABI 3730 capillary sequencer following manufacturer's instructions.

Data Analysis

Sequences were manually checked for correction with the Bioedit 7.2.5 software (Hall, 1999). All the sequences were aligned with the Clustal W method implemented in MegAlign, version 7.2 (LaserGene; DNASTAR, Madison, WI, USA). The number of haplotypes (N), haplotype diversity (*h*), and nucleotide diversity (π) per site for each species were computed with the help of DnaSP v5 (Librado & Rozas, 2009). Intra and inter-species genetic distances were calculated using the Kimura 2-parameter (K2P+G; gamma=0.744) distances (Kimura, 1980) and p-distance models in MEGA v.7 (Kumar, Stecher, & Tamura, 2016).

Species Delimitation

In present study, distance-based methods (Best Close Match, ABGD, K2P distance) and tree-based method (NJ) used for correct species identification in Anatolian *Capoeta*. Best Close Match (BCM) test in TaxonDNA/Species Identifier 1.8 (Meier, Shiyang,

Table 1. Origin and number of individuals from 21 *Capoeta* species sequenced for COI gene. Sample numbers (N), Haplotype numbers (H), Haplotype diversity (HD), Nucleotide diversity (**π**), GPS coordinates, and GenBank accession numbers.

Species, Stream, Locality, River, Drainage, Basin	N	H	Hd	π	GPS coordinates	GenBank No.
C. antalyensis						
R. Aksu at Gokdere, Mediterranean Sea Basin	5	1			37° 01.712'N / 30° 54.759'E	MH592434
	5	1				MH592435
	1	1				MH592436
	11	3	0,636±0,089	0,00112±0,00024		
C. aydinensis						
R. Banaz at Sivaslı, Usak, B. Menderes, Aegean Sea Basin	9	1			38° 32.994'N / 29° 37.224'E	MH592437
R. Kayirli at Yatagan, Mugla, B. Menderes, Aegean Sea Basin	8	2			37° 25.510'N / 28° 08.309'E	MH592437, MH592438
	17	2	0,221±0,121	0,00034±0,00019		
C. baliki						
R. Karasu at Çobanlar, Sinop, Black Sea Basin	3	1			41° 57.169'N / 35° 01.190'E	MH592439
R. Pecenek at Sereflikochisar, Ankara, Black Sea Basin	3	1			38° 51.986'N / 33° 42.415'E	MH592440
R. Abdal at Samsun, Black Sea Basin	2	1			41° 12.755'N / 36° 35.763'E	MH592440
R. Delice at Yerkoy, Yozgat, Black Sea Basin	2	1			39° 37.328'N / 34° 29.399'E	MH592440
R. Devrez at Ilgaz, Cankiri, Black Sea Basin	1	1			40° 54.277'N / 33° 38.250'E	MH592440
	11	2	0,436±0,133	0,00067±0,00020		
C. banarescui						
R. Bulanik at Savsat, Coruh, Black Sea Basin	1	1			41° 15.530'N / 42° 19.372'E	MH592442
R. Coruh at İspir, Coruh, Black Sea Basin	6	1			40° 31.656'N / 41° 02.273'E	MH592442
R. Tortum at Erzurum, Coruh, Black Sea Basin	5	1			40° 31.464'N / 41° 33.469'E	MH592442
R. Kelkit at Gümüşhane, Yeşilırmak, Black Sea Basin	4	1			40° 07.369'N / 39° 21.189'E	MH592441
R. Yesilırmak at Refahiye, Black Sea Basin	5	1			39° 55.192'N / 38° 45.593'E	MH592441
	21	2	0,514±0,046	0,00394±0,00035		
C. barroisi						
R. Afrin at Kilis, Orontes, Mediterranean Sea Basin	2	1			36° 48.368'N /36° 58.931'E	MH592443
	5	1				MH592444
	7	2	0,476±0,171	0,00073±0,00026		
C. bergamae						
R. Karabol at Kayagil, Usak, Gediz,Aegean Sea Basin	9	1			38° 37.889'N / 29° 17.774'E	MH592445
R. Koca, Biga, Çanakkale, Marmara Sea Basin	6	1			40° 16.163'N / 27° 13.172'E	MH592446
	15	2	0,510±0,069	0,0015±0,00024		
C. caelestis						
R. Goksu at Mut, Mersin, Mediterranean Sea Basin	1	1			36° 39.282'N / 33° 21.904'E	MH592447
	10	1				MH592448
	11	2	0,182±0,144	0,00028±0,00022		
C. capoeta						
R. Digor at Kars, Aras, Caspian Sea Basin	2	1			40° 15.807'N / 43° 32.346'E	MH592449
R. Selim at Kars, Aras, Caspian Sea Basin	1	1			40° 28.288'N / 42° 48.031'E	MH592449
R. Kars at Kars, Aras, Caspian Sea Basin	4	1			40° 35.705'N / 43° 03.961'E	MH592449
Deriner Dam at Artvin,Coruh Black Sea Basin	7	1			41° 10.085'N / 41° 52.168'E	MH592450
	14	2	0,538±0,052	0,00083±0,00008		

Table 1. Continued

Clade, Subclade, Species, River, Locality, Drainage	N	H	Hd	π	GPS coordinates	GenBank No.
C. damascina						
R. Asi at Serinyol, Hatay, Orontes, Mediterranean Sea Basin	5	3			36° 21.931'N / 36° 12.828'E	MH592451- MH592453
Tahtaköprü Dam (Asi) at Hatay, Orontes, Med. Sea Basin	2	2			36° 51.105'N / 36° 41.179'E	MH592451, MH592452
R. Horu at Bahçe, Ceyhan, Mediterranean Sea Basin	3	1			37° 10.661'N / 36° 29.575'E	MH592451
	10	3	0,591±0,080	0,00096±0,00044		
C. ekmekciae						
R. Coruh at Borçka, Artvin, Coruh, Black Sea Basin	9	1	0.000±0.000	0.00000±0.00000	41° 21.931'N / 41°40.458'E	MH592454
C. erhani						
R. Karaağaç, K.Maraş, Ceyhan, Mediterranean Sea Basin	2	1			37° 37.818'N / 37° 22.497'E	MH592456
R. Aksu at K.Maraş, Ceyhan, Mediterranean Sea Basin	5	2			37° 29.376'N / 36° 53.697'E	MH592455, MH592456
R. Seyhan at Karaisali, Adana, Seyhan, Mediterranean Sea Basin	4	1			37° 10.449'N / 36° 30.387'E	MH592455
	11	2	0,545±0,072	0,00084±0,00011		
C. oguzelii						
R. Ezine at Devrekani, Kastamonu, Black Sea Basin	11	1	0.000±0.000	0.00000±0.00000	41° 43.749'N / 33° 52.711'E	MH592466
C. pestai						
R. Eyilikler at Beyşehir, Isparta, Turkish Lake District	11	1			37° 55.270'N / 31° 20.653'E	MH592457
R. Çayköy at Eğirdir, Isparta, Turkish Lake District	6	2			37° 48.899'N / 30° 55.650'E	MH592457, MH592458
	17	2	0,213±0,101	0,0001±0,0001		
C. sieboldii						
Borçka Dam at Artvin, Coruh, Black Sea Basin	6	1			41° 12.191'N / 42° 01.021'E	MH592460
R. Tersakan at Havza, *Yesilirmak*, Black Sea Basin	4	1			40° 59.352'N / 35° 43.029'E	MH592459
	10	2	0,533±0,095	0,00164±0,00029		
C. tinca						
Eber Lake at Bolvadin, Afyon, Eber Lake Basin	5	1			38° 41.031'N / 31° 07.711'E	MH592461
R. Emet at Harmancık, Bursa, Susurluk, Marmara Basin	6	1			39° 40.402'N / 29° 07.855'E	MH592461
R. Sakarya at Kizilcahamam, Ankara	8	1			40° 26.964'N / 32° 39.279'E	MH592461
R. Manyas at Manyas, Balıkesir	9	1			40° 04.583'N / 27° 58.184'E	MH592461
R. Balikli at Hendek, Sakarya, Black Sea Basin	4	2			40° 46.060'N / 30° 46.260'E	MH592461, MH592462
	32	2	0,063±0,058	0,00010±0,00009		
C. trutta						
R. Ambar at Bismil, Diyarbakır, Tigris, Persian Gulf Basin	12	1			37° 53.016'N / 40° 29.143'E	MH592463
R. Murat at Mus, Euphrates, Persian Gulf Basin	7	1			38° 51.935'N / 41° 30.054'E	MH592463
	19	1	0.000±0.000	0.00000±0.00000		
C. umbla						
R. Taslicay at Taşlıcay, Agri, Euphrates, Persian Gulf Basin	1	1			39° 38.787'N / 43° 21.991'E	MH592464
R. Salat at Bismil, Diyarbakır, Tigris, Persian Gulf Basin Basin	2	1			37° 51.779'N / 40° 52.351'E	MH592464
R. Merzimen at Yavuzeli, G.antep, Euphrates, Persian Gulf Basin	2	1			37° 17.537'N / 37° 34.372'E	MH592464
R. Karasu at Araban, Gaziantep, Euphrates, Persian Gulf Basin	5	1			37° 24.835'N / 37° 37.575'E	MH592464
R. Erziki at Hakkari, Tigris, Persian Gulf Basin	3	1			37° 40.393'N / 43° 51.885'E	MH592464
R. Tohma at Gürün, Sivas, Euphrates, Persian Gulf Basin	1	1			38° 43.257'N / 37° 16.258'E	MH592464
R. Karasu, Van, Van Lake Basin	13	2			38° 49.677'N / 43° 53.653'E	MH592464, MH592465
	27	2	0,074±0,067	0,00011±0,00010		

Vaidya, & Ng, 2006) was used to select the best threshold value and to evaluate the potential of the COI dataset for correct species identification. In addition, the Automatic Barcode Gap Discovery (ABGD; Puillandre, Lambert, Brouillet, & Achaz, 2012) method was run via a web interface (ABGD web, http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) to check the distribution and size of a potential barcoding gap for COI dataset with the following settings: Pmin: 0.001, Pmax: 0.9, Step: 10, X (relative gap width):1.5, Kimura (K80), number of bins: 25. The intraspecific and interspecific K2P distances were calculated by MEGA 7.0. To check the overlap between the lowest interspecific and the highest intraspecific genetic distances, these values were plotted using the boxplot representation of R. Boxplots (Tuckey, 1977) in SPSS 21 (SPSS Inc., Chicago, IL, USA). Thus, the DNA barcoding gap, which was the maximum intra specific distance against its minimum distance to the nearest neighbour, was calculated for Turkish scrapers. Furthermore, the barcoding gap determined for *Capoeta* species was checked by verification of the 10-fold mean K2P distance rule proposed by Hebert, Stoeckle, Zemlak, and Francis, (2004). For tree-based species delimitation, Neighbour Joining (K2P; Saitou & Nei, 1987) trees of COI barcode region were inferred using uncorrected genetic distances for morphologically indistinguishable seventeen putative species in MEGA v.7 (Kumar, Stecher, & Tamura, 2016) with 1000 bootstrap replication. The test for species monophyly was performed on a NJ tree. In the NJ phylogram, it was considered that the monophyletic species were correct identification and non-monophyletic species were incorrected identification.

Phylogenetic Relationships

Maximum Likelihood (ML) and Bayesian gene trees were phylogenetically reconstructed to test the usefulness of COI barcodes in resolving phylogenetic relationships within the genus *Capoeta*. The most appropriate evolution models for the present dataset were selected using jModelTest v. 0.1.1 (Posada, 2008) with the Akaike information criterion (AIC) (Akaike, 1973) and Bayesian Information Criterion (BIC) (Schwarz, 1978). The *Capoeta* cytochrome oxidase subunit I datasets were analyzed with maximum likelihood (ML) and Bayesian inference (BI) methods. ML analysis was performed in PAUP version 4.0b10 under the predicted TrN+I+G (gamma=1.547) model with rates estimated from the data. The 100 bootstrap replicates (Felsenstein, 1985) were used to evaluate tree branch reliability in ML. The Bayesian inference analysis was implemented using MrBayes version 3.2.1 with a TrN+I model. The Markov chain Monte Carlo (MCMC) search was set to 500,000 generations, with trees sampling every 1000th generation. The common barbel, *Barbus barbus* (HQ961070), a species of the closely related *Barbus* genus, was selected as outgroup for tree-based methods.

Results and Discussion

DNA barcoding was able to delineate seventeen of the twenty-one species of scrapers from Anatolia: the remaining four of the twenty-one species showed high conspecific divergence. These four pairs of species (*C. pestai* x *C. mauricii*; *C. erhani* x *C. turani*; *C. damascina* x *C. angoreae*; *C. umbla* x *C. kosswigi*) shared their haplotypes and could not be discriminated. Based on these results, we conclude that they can be synonymized. These findings are consistent with the suggestion of Alwan et al. (2016a), Alwan et al. (2016b) and Bektas et al. (2017) that these four species pairs were simply synonymized as *C. pestai, C. erhani*, *C. damascina* and *C. umbla*. Our results also partially support previous assertions (Özdemir, 2013) that the morphological characters used to discriminate related *Capoeta* species pairs are not taxonomically reliable. The thirty-three COI haplotypes of 652 bp have been obtained for 17 *Capoeta* species distributed in Anatolia. There were no insertions, deletions and stop codons observed in the sequences. Haplotypes were submitted to the GenBank database with accession numbers MH592434-MH592466. The taxonomy, accession numbers and the site of collection are available at Table 1. Analysis of COI revealed that the whole dataset for the region contained 98 variable sites, 85 of which were parsimoniously informative. The overall transition/transversion ratio (R) was 9.54. The average nucleotide frequencies were C (29.24%), T (26.82%), A (26.53%), G (17.41%).

Genetic Divergence and Barcoding Success

The comparison of mean intraspecific and interspecific genetic distances in barcoding studies is often used to delimit species. Hebert et al. (2004) suggested that a 10-fold sequence difference between mean interspecific and mean intraspecific differences is the standard COI threshold for identification of animal species. Even if the an approximately 14-fold sequence difference between mean interspecific (4.01%) and intraspecific divergence (0.28%) based on the seventeen *Capoeta* species dataset was higher than the threshold value suggested by Hebert et al. (2004), it was observed that the inter- and intra-specific genetic range overlapped the mtDNA COI sequences obtained in this study. Consequently, there is no barcoding gap separating the intraspecific and interspecific distances. Moreover, the use of the average values instead of the largest intraspecific and the smallest interspecific distance exaggerates the size of "barcode gap" and can lead to incorrect identification (Meier, Zhang, & Ali, 2008).

Based on BCM test, the best threshold value of 1.26% was determined for the species delimitation for the mtDNA COI dataset. BCM methods with a default threshold allowed correct identification of 205 out of 253 individuals providing a 81% high success rate in these data set and 19% of all sequences (48 individuals)

are misidentified. According to the BestCloseMatch test, the overlap was found in nine species (*C. capoeta-C. ekmekciae, C. trutta-C. barroisi-C. erhani, C. tinca-C. baliki* and *C. umbla-C. damascina*) belonging to *Capoeta*genus. Therefore, *Capoeta* dataset was reduced to twelve groups based on the results of the BCM test (Figure 1). The barcoding success rate (81%) obtained for the Anatolian*Capoeta* species based on BCM test is lower than reported for Canadian freshwater fish (93%, Hubert *et al.,* 2008) and North American freshwater fish (90%; April, Mayden, Hanner, & Bernatchez, 2011). The low discriminative power of COI-based DNA barcodes in our data set can be explained by the fact that some Anatolian *Capoeta* species are young evolutionary groups (Zareian *et al.,* 2016) thought to have speciated by geological and climatic events during the Quaternary period, approximately last 2 million years (Levin *et al.,* 2012; Bektaş *et al.,* 2017). The analyses of the distribution of K2P (gamma=0.013) divergence values showed that there exists a barcoding gap (0.9-1.2%; Figure 2) between maximum intraspecific distance and minimum

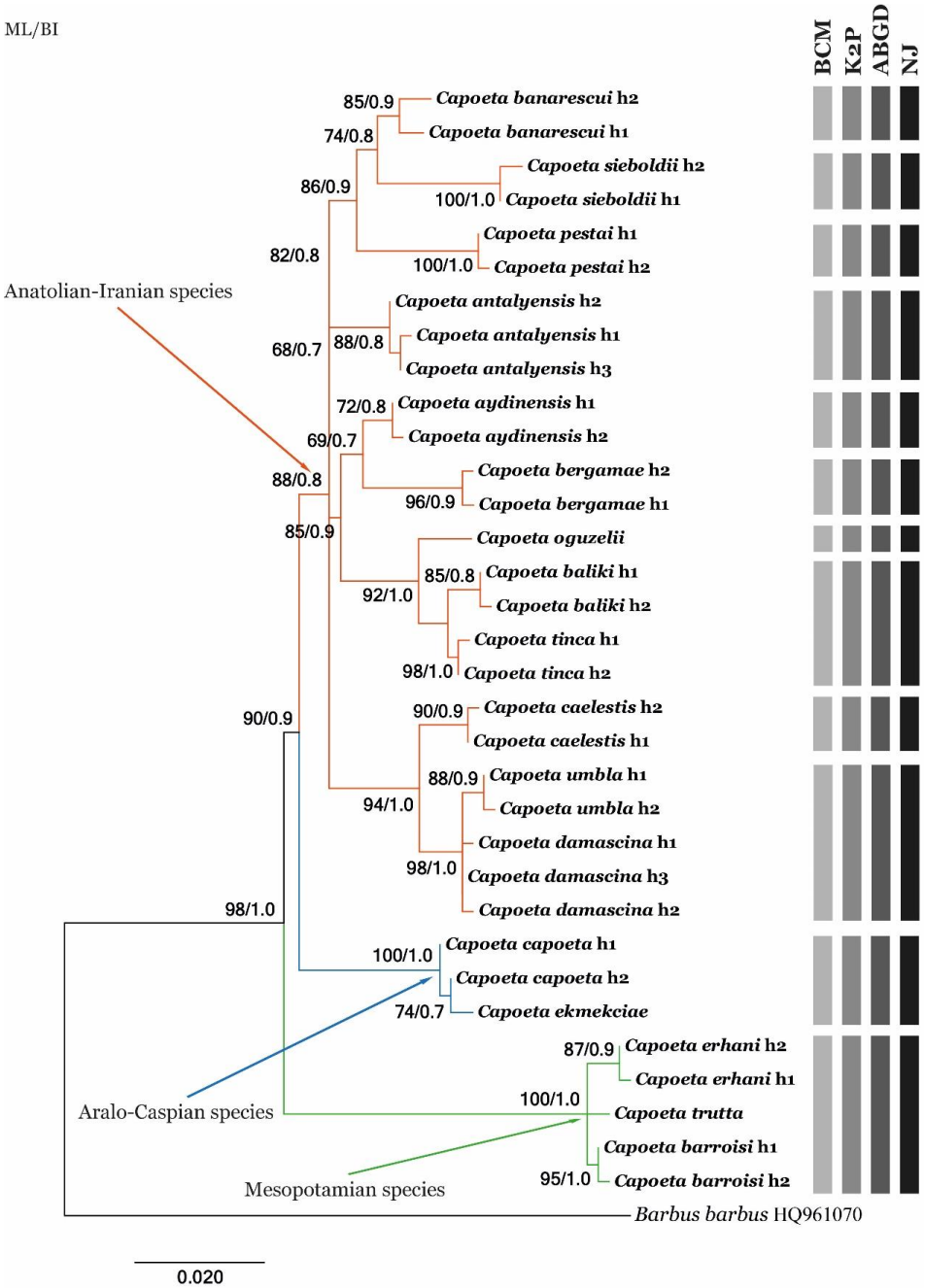
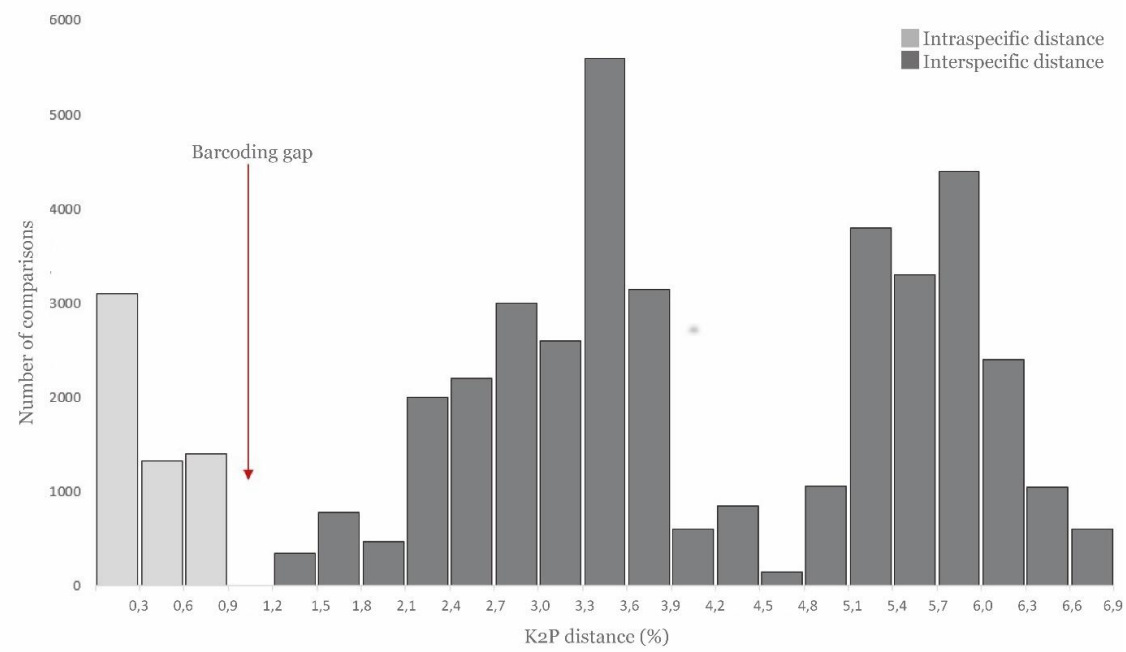
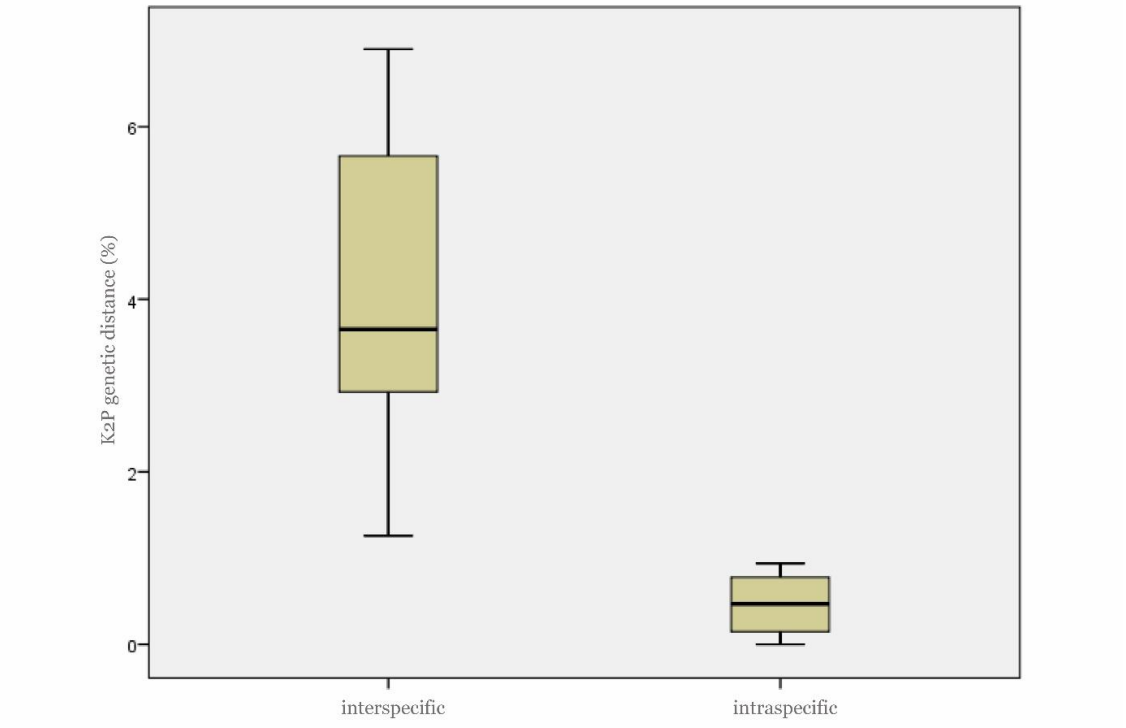


Figure 1. Summary from species delimitation analyses on a likelihood consensus tree. Node values represent likelihood bootstrap and posterior probabilities, respectively. Coloured bars represent hypothesized species groupings based on corresponding delimitation analyses based on BCM, K2P, ABGD and NJ. Coloured clades on phylogeny indicated groups of species with the same geographical tendency.

interspecific divergence values in the updated COI-dataset of Turkish scrapers. In addition, ABGD method identified twelve groups for scraper specimens in Anatolia with the initial approach and the barcode gap threshold calculated by the ABGD analysis of the COI dataset (Figure 3) was consistent with that of "K2P-based distance" and Taxon DNA's "BMC criterion" test. The Neighbour Joining (NJ, Figure 1) tree-building, which is used as a monophyly-based species delimitation method, showed twelve monophyletic groups similarity to the other delimitations results. As indicated in the maximum likelihood and Bayesian inference consensus tree (Figure 1), the individuals of seven species (*C. antalyensis, C. aydinensis, C. banarescui, C. bergamae*, *C. caelestis, C. oguzelii* and *C. sieboldii*) were clustered into a monophyletic groups that were remarkably separated from each other. These species were considered as successfully



a) Divergence frequency distribution of the COI barcode sequences for the Anatolian *Capoeta* species



b) Boxplot of the interspecific and intraspecific genetic distances, indicating the existence of a barcode gap

Figure 2. Barcoding gap.a) Divergence frequency distribution of the COI barcode sequences for the Anatolian *Capoeta* species. b) Boxplot of the interspecific and intraspecific genetic distances, indicating the existence of a barcode gap.

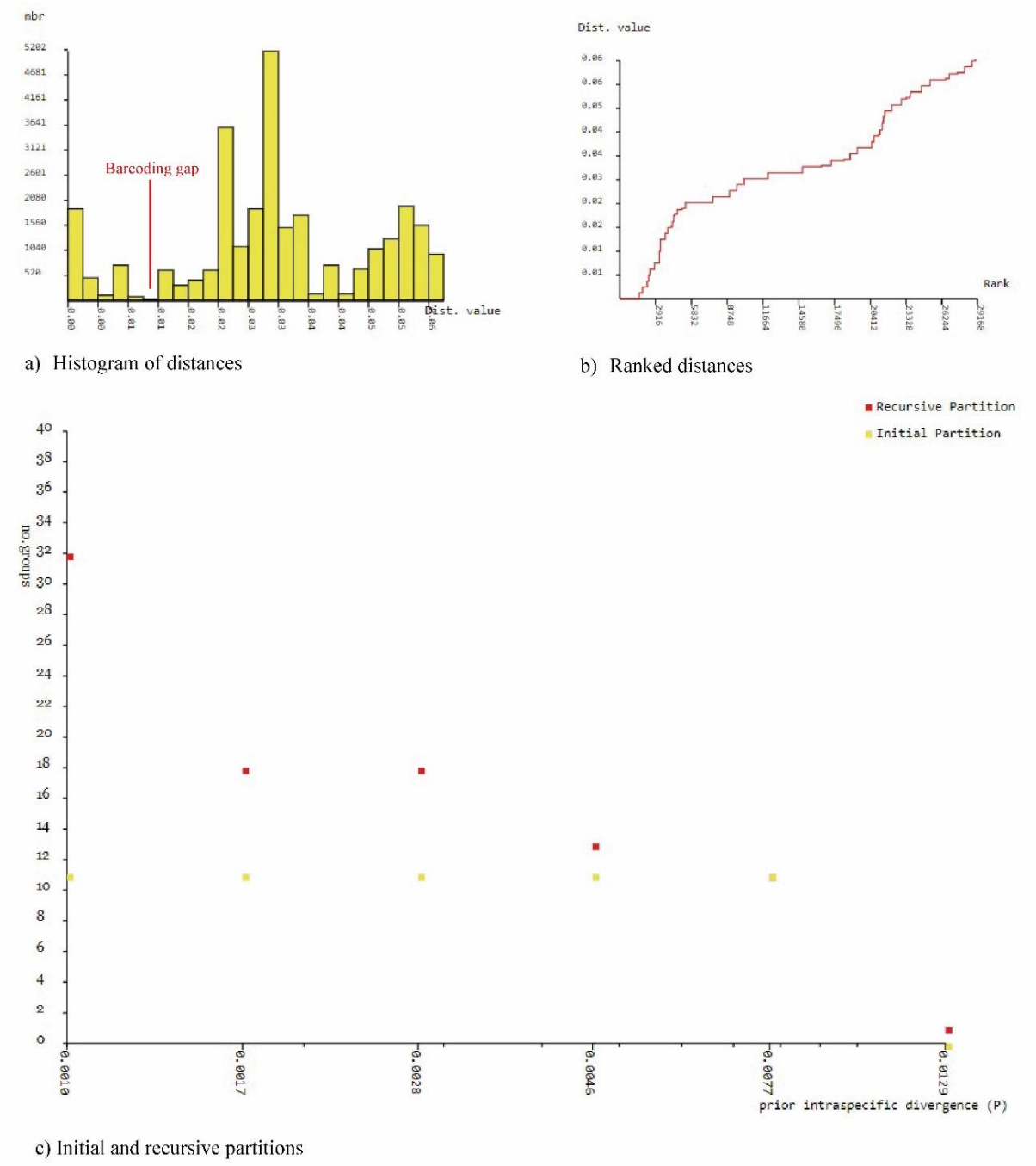


Figure 3. ABGD (Automatic Barcode Gap Discovery) species delimitation. a) Histogram of distances, b) Ranked distances, c) Initial and recursive partitions.

identified because their individuals were clustered together into a monophyletic group, which separated them from their closest relatives. However, the five species groups *C. baliki-C. tinca*, *C. damascina-C. umbla*, *C. capeota-C. ekmekciae*, *C. pestai-C. mauricii* and *C. erhani-C. barroisi-C. trutta* were clustered together (Figure 1), indicating that they were closely related. In this study, the delimiting *Capoeta* species using the COI dataset provided eleven congruent numbers of putative species (overlap was found at 5 out of 17 species) for both BCM, K2P-based distance, ABGD and NJ monophyly. Each of the groups; *C. tinca-C. baliki*, *C. capoeta-C. ekmekciae*, *C. trutta-C. erhani-C. barroisi* and *C. damascina-C. umbla* were recognized as a single cluster through the four different methods as shown on consensus phylogenetic tree (Figure 1).

Kimura's two parameter-based genetic distances were calculated for the groups identified based on these four delimitation methods. The maximum intra-specific variations (0.0094±0.0038; *trutta* complex) were less than the distance to the minimum nearest neighbour (0.0126±0.0040; *C. oguzelii*&*tinca* complex)

Table 2. Intraspecific genetic variation of *Capoeta* from Anatolian

	Species/groups name	N	H	Hd ± SD	π ± SD	Mean intra spp ± SE	Max intra spp ± SE	Nearest species	Distance to NS ± SE
1	*C. antalyensis*	11	3	0,63±0,089	0,0011±0,0002	0,0011±0,0009	0,0031±0,0023	*C. aydinensis*	0,0176±0,0053
2	*C. aydinensis*	17	2	0,22±0,121	0,0003±0,0002	0,0003±0,0003	0,0015±0,0015	*C. banarescui*	0,0159±0,0051
3	*C. banarescui*	21	2	0,51±0,046	0,0039±0,0003	0,0040±0,0018	0,0078±0,0036	*C. aydinensis*	0,0159±0,0051
4	*C. bergamae*	15	2	0,51±0,069	0,0015±0,0002	0,0016±0,0010	0,0031±0,0021	*C. aydinensis*	0,0191±0,0057
5	*C, caelestis*	11	2	0,18±0,144	0,0002±0,0002	0,0003±0,0003	0,0015±0,0015	*damascina* complex	0,0126±0,0047
6	*capeota* complex	23	4	0,77±0,035	0,0019±0,0002	0,0019±0,0010	0,0046±0,0026	*C. antalyensis*	0,0295±0,0075
7	*damascina* complex	37	4	0,46±0,082	0,0015±0,0002	0,0015±0,0009	0,0047±0,0027	*C. caelestis*	0,0126±0,0047
8	*C. oguzelii*	11	1	0,00±0,000	0,0000±0,0000	0,0000±0,0000	0,0000±0,0000	*tinca* complex	0,0126±0,0040
9	*C. pestai*	17	2	0,11±0,101	0,0001±0,0001	0,0002±0,0002	0,0015±0,0016	*C. aydinensis*	0,0226±0,0065
10	*C. sieboldii*	10	2	0,53±0,095	0,0016±0,0003	0,0016±0,0013	0,0031±0,0021	*C. banarescui*	0,0225±0,0063
11	*tinca* complex	43	4	0,45±0,080	0,0035±0,0006	0,0036±0,0013	0,0094±0,0038	*C. antalyensis*	0,0243±0,0065
12	*trutta* complex	37	5	0,68±0,064	0,0047±0,0004	0,0049±0,0019	0,0094±0,0038	*C. antalyensis*	0,0512±0,0101

Note: n/N – sample size/number of haplotypes; Hd±SD – haplotype diversity ± standard deviation; π ± SD - nucleotide diversity (per site) ± standard deviation; Mean intra-sp ± SE – mean intraspecies K2P distance ± standard error; Max intra-sp – maximum intraspecies K2P distance; Distance to NS ± SE – mean K2P+G distance to a nearest species ± standard error.

Table 3. Mean percent genetic distances betweenAnatolianspeciesof *Capoeta*under corrected K2P+G (below diagonal) and p-distance (above diagonal)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*C. tinca*		0,87	1,38	2,47	2,53	3,06	3,15	3,23	3,25	3,39	3,44	3,45	3,84	4,26	5,18	5,52	5,60	8,77
2	*C. baliki*	0,89		1,65	2,51	2,48	3,33	2,80	3,11	3,14	2,97	3,40	3,41	3,43	3,85	4,91	4,95	5,03	8,77
3	*C. oguzelii*	1,41	1,70		2,17	2,54	3,07	2,54	3,07	2,79	3,23	3,60	3,45	3,53	3,65	4,86	4,91	4,99	8,54
4	*C. aydinensis*	2,62	2,66	2,27		1,91	1,86	1,64	2,48	2,50	2,64	3,15	3,16	2,29	2,75	5,04	4,77	5,16	7,69
5	*C. antalyensis*	2,69	2,62	2,68	2,00		2,54	2,29	2,82	2,85	2,37	2,91	2,92	2,67	3,09	4,77	4,81	4,89	7,72
6	*C. bergamae*	3,27	3,57	3,26	1,93	2,68		3,16	3,38	3,40	3,39	3,75	3,76	3,84	3,96	5,94	5,98	5,76	7,92
7	*C. banarescui*	3,40	2,99	2,68	1,71	2,42	3,38		2,55	2,27	2,40	3,22	3,23	2,52	2,27	5,11	4,84	5,23	7,69
8	*C. umbla*	3,47	3,33	2,27	2,61	3,00	3,62	2,69		0,34	1,55	3,14	3,15	3,54	3,66	5,48	5,68	5,46	7,85
9	*C. damascina*	3,49	3,35	2,95	2,64	3,03	3,65	2,38	0,35		1,27	3,47	3,48	3,56	3,37	5,20	5,40	5,18	8,18
10	*C. caelestis*	3,64	3,15	3,45	2,79	2,49	3,62	2,52	1,61	1,31		3,30	3,31	3,69	3,51	5,34	5,54	5,47	8,31
11	*C. ekmekciae*	3,73	3,68	3,89	3,40	3,12	4,07	3,48	3,36	3,75	3,54		0,30	4,22	4,03	5,70	5,74	5,83	7,77
12	*C. capoeta*	3,75	3,69	3,73	3,41	3,14	4,08	3,50	3,38	3,76	3,55	0,30		4,23	4,03	5,71	5,75	5,84	7,69
13	*C. pestai*	4,22	3,71	3,82	2,42	2,85	4,19	2,68	3,83	3,85	4,00	4,67	4,69		3,34	5,48	5,21	5,61	8,61
14	*C. sieboldii*	4,69	4,18	3,93	2,91	3,31	4,29	2,38	3,94	3,61	3,76	4,40	4,42	3,60		5,45	5,06	5,57	8,69
15	*C. barroisi*	5,84	5,48	5,41	5,66	5,32	6,78	5,75	6,20	5,84	5,99	6,51	6,53	6,23	6,14		0,57	0,79	8,92
16	*C. trutta*	6,28	5,54	5,47	5,33	5,38	6,84	5,42	6,45	6,09	6,25	6,57	6,59	5,89	5,65	0,50		0,84	8,84
17	*C. erhani*	6,39	5,64	5,57	5,83	5,48	6,55	5,92	6,16	5,80	6,16	6,68	6,70	6,40	6,31	0,81	0,85		9,07
18	*B. barbus*	9,53	9,51	9,23	8,26	8,29	8,52	8,26	8,42	8,81	8,94	8,34	8,26	9,34	9,40	9,68	9,59	9,87	

(Table 2). The present results were similar to those determined in the previous study (maximum intraspecific distance ≤1.1%; Özdemir, 2013). Ward (2012) also reported that intraspecific genetic distances are based on K2P, which is less than 1%, and rarely greater than 2% in taxa including fish. Intraspecific differences in 17 *Capoeta* species based on K2P and p-distance are less than 1% given by Ward (2012). The mean genetic distances between all Anatolian *Capoeta* species using both K2P (corrected) and p-distance (uncorrected) algorithms are comparatively given in Table 3.

Consistent with the threshold value suggested by Hebert et al. (2004), the mean genetic distance between congeneric species was at least 10-fold greater than the conspecific individuals. In our present study, the approximately 14-fold difference between mean interspecific (4.01%) and intraspecific K2P genetic distance (0.28%) (Table 4) was similar to that of 17 commercially important freshwater fishes species from Turkey (approximately 19-fold for COI; Keskin & Atar, 2013b), but was lower than previous barcoding studies; for Canadian freshwater fishes (27-fold difference; Hubert *et al.,*2008), for Indian freshwater fishes (24-fold difference; Lakra *et al.,* 2016) and for Javanese-Balinese freshwater species (28-fold difference; Dahruddin *et al.,* 2017). The average congeneric variability is almost 14 fold higher than the conspecific values, and this also shows a low level of resolution between clusters in the NJ tree to group the conspecific individuals to their corresponding monophyletic species (Figure1). Consistent with the hypothesis of vicariant speciation, the Anatolian *Capoeta* species were allopatrically distributed and their areas of distribution correlate with phylogenetic relationships similar to some other Anatolian freshwater fish species (Hrbek et al. 2002; Hrbek et al. 2004).

Phylogenetic Analysis

COI-based DNA barcodes were produced from *Capoeta* individuals sampled at their type localities as much as possible (Table 1). The topologies of the maximum likelihood and Bayesian inference consensus trees of COI-barcode region sequence data analyses were highly similar, and revealed three main clades within the genus *Capoeta* in Anatolia with high bootstrap support (ML/BI: 98/1.0; Figure 1) according to their geographical origin as Anatolian-Iranian species (*C. antalyensis, C. aydinensis, C. baliki, C. banarescui, C. bergamae, C. caelestis, C. damascina, C. oguzelii, C. pestai, C. sieboldii, C. tinca* and *C. umbla*; Clade I), Aralo-Caspian species (*C. capoeta, C. ekmekciae*; Clade II) and Mesopotamian species (*C. trutta*, *C. barroisi* and *C. erhani*; Clade III). The present results were in congruence with the molecular phylogeny of the Anatolian *Capoeta* species suggested by Özdemir (2013). Contrary to the taxonomic classifications previously proposed, the phylogeny of Anatolian *Capoeta* species based on mtDNA 16S rRNA or cytochrome *b* sequences (Turan, 2008; Zareian et al. 2016; Bektaş *et al.,* 2017), was consistent with previous COI-based genetic studies (Levin *et al.,* 2012; Alwan et al. 2016; Ghanavi et al. 2016). Therefore, the presence of three *Capoeta* lineages on the phylogenetic trees based on COI data can be more easily explained by their geographical distribution instead of their morphological characteristics.

Conclusion

The present results confirmed that the integrated use of DNA barcoding (BCM, K2P distance, ABGD and NJ species delineation) are efficient and reliable methods for delineation and genetic identification of the Anatolian *Capoeta* species. Due to the presence of low level of pairwise intra-specific divergence between the nearest neighbour species, the presence of some putative *Capoeta* species in the Anatolian freshwater fish fauna could not be confirmed based on the COI barcode sequences. However, for the most of analysed species, a so-called 'barcoding gap' exists: intraspecific sequence divergence levels are clearly lower than interspecific divergence to the nearest neighbour taxon in the dataset. This research of the *Capoeta* expands the DNA barcode database and provides benchmark data for future studies.

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Table 4. Distance summary based on corrected K2P and uncorrected p-distance models within and between *Capoeta* species.

Model	Level	Min dist (%)	Mean dist (%)	Max dist (%)	SE dist (%)
K2P	Within species	0,00	0,28	0,94	0,01
	Within genus	1,26	4,01	6,90	0,08
p- distance	Within species	0,00	0,27	0,92	0,01
	Within genus	1,23	3,66	5,98	0,07

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