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Morphological and Molecular Perspective on Geographical Differentiation of *Barbus* Populations (Actinopterygii; Cyprinidae) within Iranian Freshwater Drainages

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Abstract

Geographically isolated populations of *Barbus* present remarkable variation with regard to morphometric and meristic characters within Iranian drainage systems; while they are relatively similar in external morphology such as coloration. Present study is the first report on geographical differentiation of *Barbus* populations in Iran from both morphological and molecular point of views. The morphometric and meristic characters as well as phylogenetic relationships (using sequence data of mtDNA cytochrome *b* gene) of twelve populations from three drainages (i.e. South Caspian Sea, Orumyieh and Tigris-Karoun) were analyzed. The univariate analysis reveals significant morphological differentiation among the populations from the three drainages. The phylogenetic analyses indicate relatively high genetic differentiation among the populations from the mentioned drainages, which are consistent with the observed morphological variation, and correspond well with their biogeographic distribution. However, the result of Automatic Barcode Gap Discovery (ABGD) analysis shows intra-specific differentiation for the sequences from South Caspian Sea drainage, while support the distinction of the specimens from Tigris-Karoun drainage as *B. lacerta*. Since the sequence data of *Barbus cyri* from Armenia is clustered with individuals from the South Caspian Sea and the morphological comparison, the specimens from that basin are considered as *B. cyri* and the Orumyieh basin as *Barbus* sp.

Keywords: Biogeography, geographical variation, geological history, zoogeography, taxonomic units.

Introduction

It is well known that genus *Barbus* Cuvier and Cloquet (1816), shows very wide range of distribution including the Europe and West Asia (Kottelat and Freyhof, 2007). It also includes high numbers of species in the Middle East (Berrebi *et al.*, 1996).

The Middle East, particularly Iranian Plateau has been considered as a principal zoogeographic interchange area for the freshwater ichthyofauna during the gradual closing of the Tethys Sea (Durand *et al.*, 2002; Coad, 1987, 1998). Therefore, the present Iranian freshwater ichthyofauna is a combination of the elements from Euromediterranean (Perea *et al.*, 2010) as well as African regions (Coad and Vilenkin, 2004). The particular examples are species of the genus *Barbus* that considered being the most diversified group among the Iranian cyprinids (Esmaeili *et al.*, 2010).

Among the species of *Barbus* in Iran, *Barbus cyri* (Filippi, 1865) appears to show the widest distribution. Its populations have been recorded from South Caspian Sea (Berg, 1949; Bianco and Banarescu, 1982; Kiabi *et al.*, 1999; Abdoli, 2000), (Figure 1). In addition, *Barbus lacerta* has been recorded from Tigris basin and its type locality is Kueik River, Aleppo. We compared the specimens from Tigris-Karoun with Syntype-Aleppo of *B. lacerta* and concluded that the specimens from Tigris-Karoun belong to *B. lacerta*.

Based on several lines of evidence, these populations represent remarkable differences with regard to morphometric characters such as head length, body depth and size of eye and mouth (Almaça, 1983, 1986; Karaman, 1971; Bianco, 1995; Saadati, 1977; Motamedi, 2010), while they are relatively similar in external morphology (e.g. coloration).

In addition to morphometric characters, the previous studies have pointed out that distinct differences exist among the isolated populations with regard to the meristic characters such as the number of pectoral and dorsal fin rays, the number of gill rakers and lateral line series (e.g. Berg, 1948-1949; Saadati, 1977; Motamedi, 2010). However, little is known whether these variations are related to

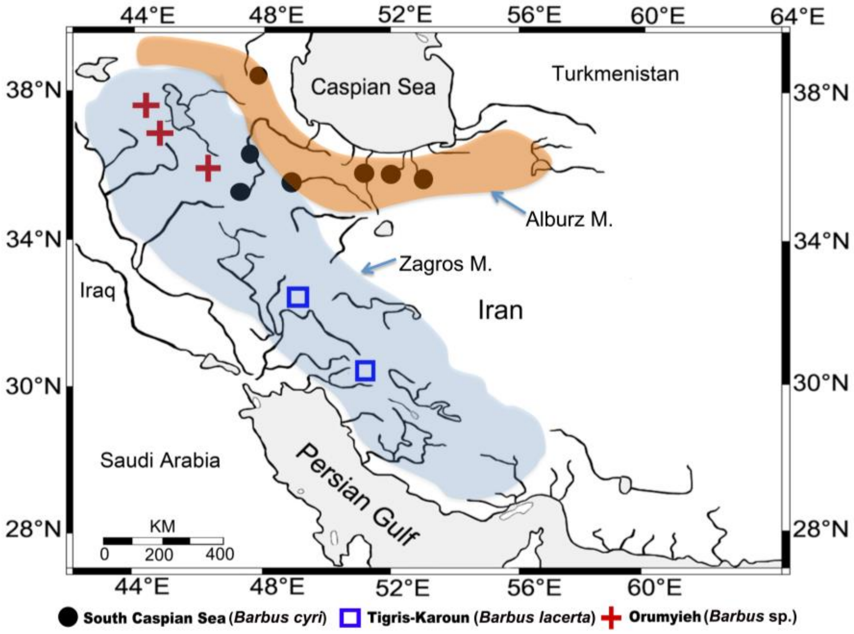

Figure 1. Map of Iran shows position of Zagros and Alburz mountains and also the drainage systems from which the populations were sampled.

ecological factors (eco-plasticity) in different environments, genetic divergence because of geographic speciation or to combination of both factors.

Based on the total evidence approach, a wide range of distribution with high morphological variation proves these species requiring revision. Some of the synonyms may be distinct, while populations in isolated basins in Iran and elsewhere could be new taxa (Abdoli, 2000; Esmaeili *et al.*, 2010; Coad, 2013). Therefore, the previous authors have strongly suggested investigation of the populations based on both morphological and molecular data (Coad, 2013).

The aim of this study is to contribute to understand the phenotypic and genetic variations between the populations of the above mentioned species from three drainages in Iran i.e. Orumyeih, South Caspian Sea and the Tigris-Karoun.

We studied a large data set including both morphological characters and mtDNA sequences (cytochrome *b* gene) of the populations from three drainages to understand whether morphological and molecular characters show consistent pattern of variation among the populations from the three drainages and also to discuss the possible causes of phenotypic variations. We aimed additionally to evaluate taxonomic status of the populations from the mentioned drainages.

Materials and Methods

Localities, Examined Specimens

One hundred seventy nine specimens (158 for morphological and 21 for molecular analyses) belong to 12 localities were collected from their representative drainage systems i.e.; South Caspian Sea (seven localities), Tigris–Karoun (two localities) and Orumyieh (Figure 1 and Table 1). Additional materials consist of six syntypes from Aleppo (NMW 54158-1-6) and five specimens from the Tigris drainage in Turkey (94738-1-5) from Naturhistorisches Museum Wien (NMW) were also included for the morphological analyses. These later specimens were not in a good condition of preservation, and we therefore use them only for comparison of the meristic characters. All the collected samples are deposited in the Zoological Museum of the Biology Department at Shiraz University (ZM–CBSU) and the Zoological Museum of Shahid Bahonar University of Kerman (ZM–SBUK) Iran.

Morphological Analysis

Thirty one morphometric characters were measured using vernier caliper with an accuracy of 0.05 mm and six meristic characters were counted using standard methods (Table 2 for characters and the abbreviations). Measurements and abbreviations follow Holcik (1989). Measurements of the morphometric characters were standardized in order to eliminate any size effect (Lahnsterner and Jagsch, 2005): Standardized measurement = (Mmp/SL*100); Mmp: measured morphometric parameter, SL: standard length. In addition to the measurements, which are given as percentage of SL, other measurements are presented as percentage of Prad (preanal distance) and HL (head length). In total, 57 standardized morphometric characters were calculated and applied for the statistical analyses.

Table 1. Sampling sites and the collection details

Sampling site and Collection details	N	Drainage basin	SL mm (Mean± S.D.)
Chaisalman River	12	South Caspian Sea	110.25±2.68
Idaghamush River* (n=1, M72)	10	South Caspian Sea	78.05±1.12
Ghezel Ozan River	10	South Caspian Sea	107.10±2.55
Tajan River* (n=4, M62, M63, M74, M622)	14	South Caspian Sea	93.55±1.34
Talar River* (n=3, M70, M71, M75)	13	South Caspian Sea	68.10±2.12
Abhar River* (n=1, M76)	9	South Caspian Sea	143.00±3.78
Divandarreh River* (n=2, M66, M67)	9	South Caspian Sea	58.25±2.02
Babolrud River * (n=4, M68, M73, M107, M682)	-	South Caspian Sea	63.75±3.10
Kashkan River	15	Tigris-Karoun	107.95±1.36
Bashar River* (n=4, M108, M109, M110, M111)	26	Tigris-Karoun	99.65±4.25
Serudan River * (n=2, M64, M65)	12	Orumyieh	118.50±2.65
Nazluchai River	16	Orumyieh	65.35±2.79
Orumyieh River	12	Orumyieh	131.40±3.22

*: The populations that are used for molecular analysis.
S.D.= standard deviation, n=number of specimens for molecular analysis and is followed by the specimen's code;
N=number of specimen for morphological analysis. Because of the low number of specimens from Babolrud River, we used them only in molecular analysis.

The Kolmogorov-Smirnov test was applied to infer possible sexual dimorphism between the individuals. Univariate analysis of variance (ANOVA) with Duncan's multiple range test as a *Post-hoc* was carried out to test the significance of morphometric and meristic variations among the populations. The Canonical Discriminant Analysis (CDA) was used for multivariate analyses to show the classification success. Furthermore, Principal Components Analysis (PCA) was performed to reduce the dataset (Veasey *et al.*, 2001), and to extract the most weighted characters of the populations from three drainages. The phenotypic relationships between the populations was demonstrated by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram of cluster analysis, which is based on the data of Euclidean distances (Similarity measure) using the between groups linkage method. All the statistical analyses were carried out using PASW 20.00 (SPSS Inc, 2012) and PAST (PAlaeontological STatistics, ver. 1.81, Hammer *et al.*, 2001).

DNA Extraction, Sequencing and Molecular Phylogenetic Analysis

The genomic DNA was extracted from fin clips of the specimens preserved in 96% ethanol. The fin clips were digested with Tris-HCl ⁄ EDTA and NaOH / SDS solution, followed by phenol chloroform extraction (Sambrook *et al.*, 1989). The quantity of the extracted DNA was calculated with NanoDrop at the absorption of 260 nm and its quality was checked by 0.8% agarose-gel-electrophoresis.

The mitochondrial cytochrome *b* gene was successfully amplified by polymerase chain reaction (PCR) using the primers GluF (5′ AACCACCGTTGTATTCAACTACAA 3′) and ThrR (5′ ACCTCCGATCTTCGGATTACAAGACCG 3′, Machordom and Doadrio, 2001). The amplification process was performed as follow; initial denaturation 94°C (2 min), 35 cycles with denaturation at 94°C for 45 s, annealing at 58°C for 1 min, extension at 72°C for 90 s per cycle and a final extension phase at 72°C for 5 minutes.

The PCR mixtures were prepared in a final volume of 25 µl containing 10.8 µl ddH$_2$O, corresponding 5 mM buffer, 3 mM MgCl$_2$, 2 mM BSA, dNTPs, 1 µl primer, 0.2 mM *Taq* DNA polymerase and 1–2 µg DNA. The negative and positive controls were performed for all the reactions.

Sequencing was performed by Macrogen Company, South Korea. Two additional sequences were obtained from the NCBI GenBank (http://www.ncbi.nlm.nih.gov) and included in the analyses; i.e. *Barbus lacerta cyri* from the Kura basin, Armenia (accession number AF145936) and *B. lacerta* from the Tigris basin, Turkey (accession number AF145935). *Luciobarbus mursa* (accession number AF145943), *L. albanicus* (accession number AF112126) and *Carasobarbus luteus* (accession number AF145944) were used as outgroups.

Sequences were trimmed and assembled in Geneious 5.6 (Biomatters) and subsequently aligned using the Muscle 3.6 (Edgar, 2004), as incorporated in Geneious.

Bayesian analyses of nucleotide sequences were run with the parallel version of MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) under the most generalizing model (GTR+G+I) because overparametrization apparently does not negatively affect Bayesian analyses (Huelsenbeck and Ranala, 2004). Maximum likelihood reconstructions were performed using RAxML 7.2.5 (Stamatakis, 2006) under the GTR model of nucleotide substitution with CAT approximation of rate heterogeneity and fast bootstrap (1000 bootstrap replicates).

We also applied the recently described bioinformatics tool, Automatic Barcode Gap Discovery (ABGD, Puillandre *et al.*, 2012) for species delimitation analysis. ABGD automatically detects the breaks in the distribution of genetic pairwise distances, and uses them to partition the data. The

Table 2. Morphometric and meristic characters with abbreviation used in the text

Character No.	Abbreviation	Characters
Morphometric characters		
1	TL	Total length
2	FL	Fork length
3	SL	Standard length
4	HL	Head length
5	HD	Head depth
6	HW	Head width
7	Prod	Preorbital distance
8	Psod	Postorbital distance
9	Inod	Interorbital distance
10	ED	Eye diameter
11	Prdd	Predorsal distance
12	Psdd	Postdorsal distance
13	Ldf	Length of dorsal fin
14	Ddf	Depth of dorsal fin
15	Laf	Length of anal fin
16	Daf	Depth of anal fin
17	Prad	Preanal distance
18	Lpcf	Length of pectoral fin
19	Lplf	Length of pelvic fin
20	PplL	Prepelvic length
21	Maxbd	Maximum body depth
22	Minbd	Minimum body depth
23	Dpcaf	Distance between pectoral and anal fins
24	Dpcplf	Distance between pectoral and pelvic fins
25	Dplaf	Distance between pelvic and anal fins
26	Lcauf	Length of caudal fin
27	Lcaup	Length of caudal peduncle
28	Lb1	Length of barbel 1
29	Lb2	Length of barbel2
30	BW	Body width
31	MW	Mouth width
Meristic characters		
1	Dfr	Numbers of dorsal fin branched rays
2	Afr	Numbers of anal fin rays
3	Pcfr	Numbers of pectoral fin rays
4	Plfr	Numbers of pelvic fin rays
5	GR	Numbers of gill rakers
6	LL	Numbers of lateral line series scales

same species/or populations therefore should be grouped in the same partition. More specifically, ABGD act based on the similarity criterion that genetic distances between specimens from close species are supposed to be lower than genetic distances between specimens from different species (Hebert *et al.*, 2003). Therefore, ABGD analysis can be used as a primary glance for the species identification that is, primary species hypotheses (PSHs, Goldstein and DeSalle, 2011).

Results

Phenotypic Variation between Three Drainages

No significant differences were detected between the males and females with regard to the morphometric and meristic characters (Kolmogorov–Smirnov test, P>0.05). Therefore, the univariate and multivariate analyses applied for the whole data sets (males and females together). Based on the univariate analysis, the following characters show significant differences among the three drainages: length of dorsal fin/standard length, length of dorsal fin/preanal distance, maximum body depth/standard length, maximum body depth/preanal distance, the number of pelvic fin rays and the number of gill rakers (ANOVA, Duncan *Post-hoc* test, P<0.05).

The Canonical Discriminant Function Analysis (CDA), which performed on 63 morphometric plus meristic characters, shows high overall classification success among three species from three drainages (97.5%) (Figure 2 and Table 3).

The Principal Components Analysis (PCA) was carried out for all the morphometric characters and shows that the first seven PCs (out of 17) accounted for 61.60% of overall variance of the dataset and explained the most variability (Figure 3). The most weighted morphometric characters (i.e., 22 characters with eigenvalue of >0.640) were selected and ANOVA with *Post-hoc* test (P<0.05) was carried out (Table 4). The result reveals significant differences

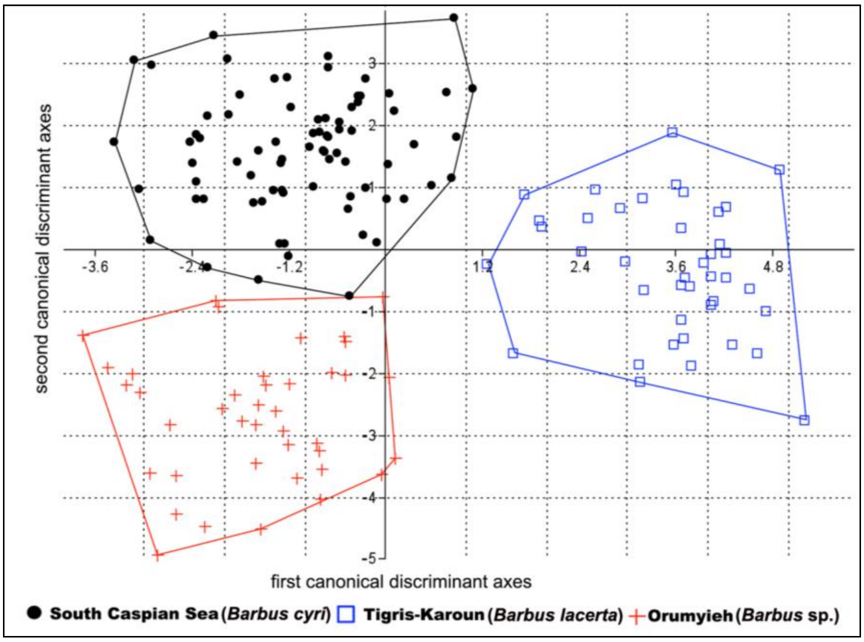

Figure 2. Discriminant function scores based on all the morphometric and meristic characters for the populations from the three drainages.

Table 3. Jackknifed classification matrix of the canonical discriminant analysis based on morphometric plus meristic characters for populations from three drainages

Drainage system	Predicted Group Membership			
	South Caspian Sea	Orumyieh	Tigris-Karoun	Total
South Caspian Sea	87.0 (67)	10.4 (8)	2.6 (2)	77
Orumyieh	10.0 (4)	90.0 (36)	0.0 (0)	40
Tigris-Karoun	0.0 (0)	0.0 (0)	100.0 (41)	41

The percentage in rows represents the classification into the groups given in columns; the corresponding number of specimens is given in brackets. The percentages of correctly classified individuals are in bold. Overall classification success is 97.5% (Wilks'λ = 0.169).

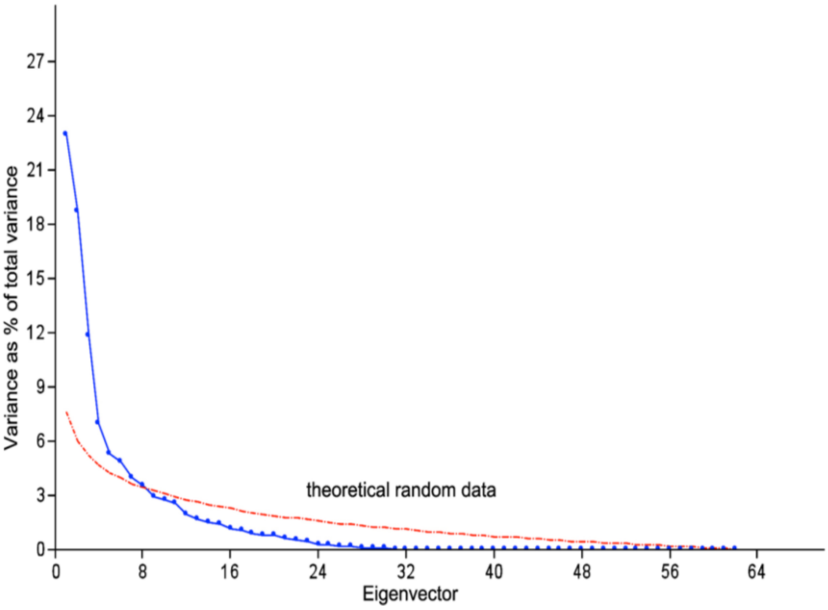

Figure 3. Eigenvector versus variance as percentage of total variance plot showing the position of the eigenvalues of the eigenvectors with respect to theoretical eigenvalues expected by chance alone.

between the mean values of 12 morphometric characters among the three drainages (Table 5).

The UPGMA dendrogram of cluster analysis was performed for two data sets separately (i) all the morphometric characters plus meristic characters and (ii) the most weighted morphometric and meristic characters, which are selected by the PCA analysis (Figure 4). Both dendrograms illustrate consistent pattern of phenotypic relationships and categorized populations into three groups, in which the individuals from Tigris-Karoun drainage are separated from the two others. It also shows that the Orumyieh specimens are morphologically close to those from the South Caspian Sea basin (Figure 4).

As a result, the present morphological data demonstrate a clear separation of the studied populations from Tigris-Karoun drainage, while Orumyieh and South Caspian Sea drainages are morphologically slightly closer each other.

Comparison of Meristic Characters

The univariate analysis of variance based on the mean of meristic characters shows that the number of gill rakers and of the lateral line series scales are slightly different among the studied populations from the three drainages (Table 6).

Phylogenetic Relationships

The resulting trees from Maximum Likelihood and Bayesian Inference methods are largely congruent and show relatively the same pattern of clustering for the studied populations (Figure 5). Moreover, ABGD analysis using JC69 Jukes–Cantor measure of distance was performed on sequences of the studied populations from the three drainages as follow; initial partition and the final recursive partition with prior maximal distance P=5.99e-02 and P=1.00e-03 respectively, and distance JC69 Jukes-Cantor

Table 4. PCA analysis of the morphometric characters for the populations from three drainages. The values for the most weighted characters in each component (the values with eigenvalue of >0.640) are given in bold

Characters	PC1	PC2	PC3	PC4	Characters	PC1	PC2	PC3	PC4
TL.SL	0.33	0.16	-0.34	0.05	HL.Prad	0.72	-0.13	0.26	0.10
FL.SL	0.35	0.05	-0.10	0.10	HD.Prad	0.18	0.27	0.25	0.01
HD.SL	0.14	-0.15	-0.09	0.11	Prod.Prad	0.77	-0.06	0.18	0.26
HL.SL	0.22	0.26	-0.13	0.02	Psod.Prad	0.51	0.07	0.17	0.04
ED.SL	0.68	-0.04	-0.03	0.09	ED.Prad	0.67	-0.03	0.06	0.09
Prod.SL	0.40	-0.07	-0.10	0.27	Inod.Prad	0.02	0.77	0.23	-0.07
Psod.SL	0.53	0.04	-0.20	0.04	Prdd.Prad	0.58	0.24	0.41	0.04
Inod.SL	0.05	0.77	-0.07	-0.07	Psdd.Prad	0.30	0.03	0.39	-0.01
Ldf.SL	0.03	0.12	-0.08	-0.03	Ddf.Prad	0.70	-0.24	0.28	0.06
Laf.SL	-0.02	0.15	-0.10	-0.12	Ldf.Prad	0.00	0.12	0.17	-0.03
Ddf.SL	0.74	-0.26	-0.02	0.07	Laf.Prad	-0.03	0.16	0.09	-0.12
Daf.SL	0.18	-0.03	-0.08	0.74	Daf.Prad	0.16	-0.01	0.15	0.73
Prad.SL	0.08	-0.04	-0.96	0.01	Lpcf.Prad	0.73	0.18	0.11	0.19
Prdd.SL	0.61	0.20	-0.25	0.05	Lplf.Prad	0.74	0.01	0.17	0.15
Psdd.SL	0.36	0.01	-0.14	-0.00	Maxbd.prad	0.11	0.65	0.12	-0.06
Lpcf.SL	0.75	0.16	-0.24	0.19	Minbd.prad	-0.05	0.71	0.25	-0.09
Minbd.SL	-0.02	0.72	-0.06	-0.09	Dpcplf.Prad	-0.21	0.21	0.16	-0.04
Maxbd.SL	0.13	0.65	-0.14	-0.06	Dplaf.Prad	-0.10	0.12	0.20	-0.11
Dpcplf.SL	-0.17	0.19	-0.26	-0.04	Lcf.prad	0.60	0.01	0.13	0.01
Dplaf.SL	-0.06	0.10	-0.23	-0.11	Lcauf.Prad	-0.07	0.05	0.74	-0.03
Lplf.SL	0.75	-0.00	-0.21	0.15	MW.Prad	0.15	0.08	0.08	0.17
Lcaup.SL	-0.05	0.04	-0.04	-0.04	Lb1.Prad	0.14	-0.21	0.14	0.27
Lcauf.SL	0.66	-0.00	-0.13	0.01	Lb2.Prad	0.11	0.02	0.02	0.37
MW.SL	0.17	0.07	-0.12	0.16	HD.HL	-0.49	0.34	-0.02	-0.07
Lb1.SL	0.15	-0.21	-0.00	0.18	Prod.HL	-0.25	0.06	-0.00	0.23
Lb2.SL	0.13	0.02	-0.13	0.26	Pood.HL	-0.36	0.27	-0.09	-0.03
FL.Prad	0.21	0.08	0.17	0.07	ED.HL	0.33	0.01	-0.01	0.07
TL.Prad	0.26	0.20	0.46	0.04	Inod.HL	-0.51	0.13	0.01	-0.12
SL.Prad	-0.08	0.04	0.96	-0.00					

Table 5. Mean and standard deviation (Mean ± S.D.) for the morphometric characters among the three drainages, which are selected by PCA

	South Caspian Sea (n=77)		Orumyieh (n=41)		Tigris-Karoun (n=40)	
Characters	Min-Max	Mean± S.D.	Min-Max	Mean± S.D.	Min-Max	Mean± S.D.
Ed.SL	2.90-6.10	4.13±0.70	2.59-6.29	4.77±1.84	2.80-8.44	4.24±1.02
Inod.SL$^{**, ***}$	7.61-10.70	8.37±0.68	7.08-9.88	7.66±0.54	6.32-8.88	8.21±0.58
Ddf.SL	14.22-25.14	19.34±1.73	15.68-23.38	19.61±1.32	16.53-22.08	19.14±1.92
Daf.SL****	14.25-23.45	17.88±1.85	14.64-23.10	18.09±2.07	13.49-22.62	19.20±2.19
Prad.SL	67.22-79.45	72.50±1.75	69.23-77.80	72.02±2.69	65.86-81.02	72.68±1.78
Lpcf.SL	15.04-20.55	18.35±1.21	15.27-22.00	18.23±1.39	16.28-21.08	18.77±1.67
Minbd.SL$^{**, ***}$	8.47-12.24	10.31±0.80	7.71-11.47	9.51±0.71	8.89-11.79	10.03±0.75
Maxbd.SL$^{**, ***}$	14.93-24.64	20.85±1.71	17.66-22.96	18.12±1.73	14.67-23.72	20.09±1.35
Lplf.SL	13.84-18.67	16.13±1.09	13.68-18.77	16.04±0.92	14.48-18.00	16.14±1.38
Lcauf.SL$^{*, **}$	19.65-29.02	24.04±2.11	16.75-30.32	23.06±2.05	18.46-28.76	23.58±3.26
SL.Prad****	125.8-146.6	138.00±3.29	128.3-144.3	139.02±5.08	123.4-151.8	137.65±3.34
HL.Prad$^{**, ***}$	30.26-40.90	34.63±2.22	27.62-40.89	36.28±2.44	31.66-42.76	34.98±3.42
Prod.Prad**	11.71-17.51	14.48±1.25	11.08-16.61	15.53±1.34	12.50-19.05	14.16±1.35
ED.Prad$^{**, ***}$	3.92-8.28	5.69±0.95	3.49-8.40	6.62±2.54	4.00-11.72	5.84±1.39
Inod.Prad$^{*, **}$	9.74-14.97	11.55±0.94	9.66-13.27	10.65±0.80	8.72-12.08	10.30±0.88
Ddf.Prad	19.71-33.90	26.69±2.38	21.28-32.27	27.24±1.86	23.08-32.24	26.35±2.76
Daf.Prad****	19.51-32.34	24.67±2.47	19.88-32.84	25.13±2.90	18.61-32.18	26.45±3.24
Lpcf.Prad	21.16-28.93	25.32±1.63	21.40-31.40	25.31±1.73	22.15-28.89	25.84±2.42
Lplf.Prad	18.91-25.77	22.24±1.40	18.25-26.16	22.28±1.25	19.88-25.74	22.21±1.90
Maxbd.Prad****	21.24-33.08	28.76±2.29	23.80-31.08	25.19±2.57	18.11-33.25	27.66±2.07
Minbd.Prad	11.40-17.21	14.23±1.13	10.23-16.22	13.22±1.10	11.13-16.52	13.81±1.13
Lcauf.Prad	27.49-39.11	33.16±2.81	23.33-43.56	32.02±2.72	26.01-41.26	32.45±4.57

(*) shows significance between South Caspian Sea vs. Tigris-Karoun;
(**) shows significance between South Caspian Sea vs. Orumyieh;
(***) indicates significance between Tigris-Karoun vs. Orumyieh;
(****) refers to significance among the three drainages (ANOVA, Duncan test, $P<0.05$).
S.D. refers to standard deviation and N refers to number of specimens. See Table II for abbreviation.

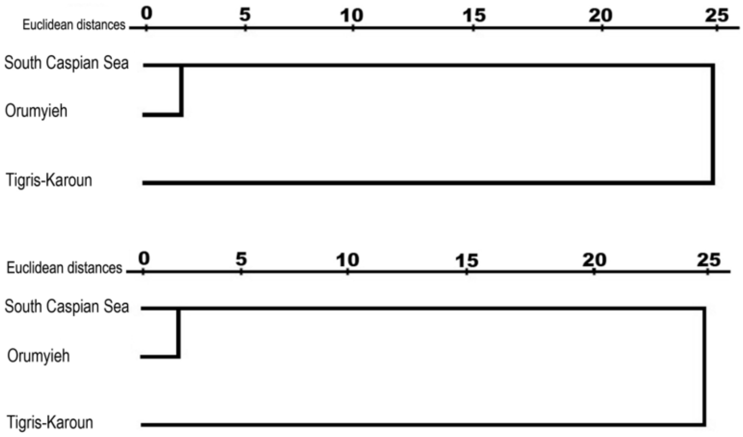

Figure 4. UPGMA dendrogram of hierarchical cluster analysis based on Euclidean distances using average linkage represents phenotypic relationships among the populations from three drainages. The dendrogram is created based on all the morphometric and meristic characters (above) and the most weighted morphometric and meristic characters, which are selected by the PCA factors (below).

MinSlope=1.500000.

Maximum Likelihood and Bayesian Inference analyses show that the Tigris-Karoun individuals form a monophyletic group (*Barbus lacerta*). It is sister to another clade that contains the individuals from Orumyieh plus South Caspian Sea drainages. Moreover, the individuals from Orumyieh plus Turkey (*Barbus* sp.) together represent distinct clade sister to the South Caspian Sea (*Barbus cyri*) (Figure 5).

Based on the ABGD analysis, the sequences from Orumyieh drainage (M65 + M64, partition VI) are grouped in the same partition, and followed by the sequences from Tigris-Karoun drainage (M108 + M109 + M110 + M111, partition VII). Most of the *Barbus cyri* sequences from the Southern Caspian Sea

Table 6. Descriptive comparison of the number of gill rakers (GR) and the number of lateral line series scales (LL) of the materials from Iran, Turkey as well as the syntype specimens from Aleppo

Drainage system	Character	N	Min.	Max.	Mean	S.D.
South Caspian Sea	LL	77	51	74	60.43	5.23
	GR		7	10	8.25	0.86
Orumyieh	LL	40	59	84	70.03	6.01
	GR		6	10	7.27	0.87
Tigris–Karoun	LL	41	61	78	68.02	4.02
	GR		8	11	9.07	0.75
Turkey	LL	6	68	78	74.2	3.76
	GR		9	10	9.63	0.02
Syntype-Aleppo	LL	10	57	71	63.10	3.64
	GR		10	12	11.21	0.03

S.D. refers to standard deviation and N refers to number of specimens.
Min. and Max. indicate respectively minimum and maximum range for the meristic characters.

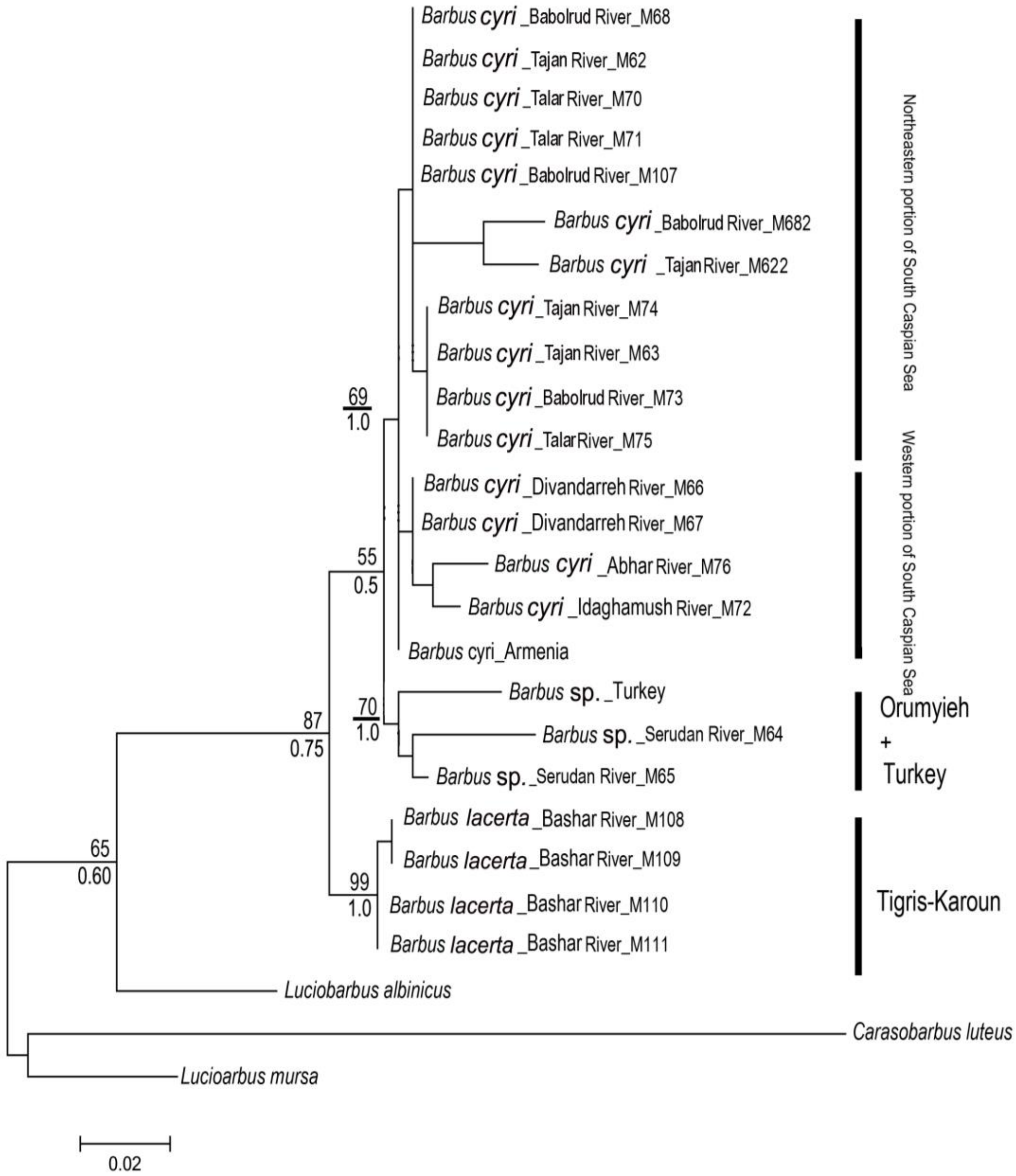

Figure 5. Maximum likelihood and Bayesian likelihood estimation of the phylogenetic relationships of the populations from three drainages in Iran. Numbers above nodes refer to the bootstrap support values followed by the posterior probabilities based on 2000 replicates. The term after species name in each branch refer respectively to the sampling site and specimen code in this study.

drainage are grouped in the same partition. However, they show intra-drainage differentiation (i.e. M70 (partition IX), M68 (partition IV), M62 (partition V), M67 + M682 + *B. cyri* from Armenia + M63 + M66 + M71 + M72 + M73 + M74 + M75 + M76 + M107 + M622 (partition X)). This finding is consistent with the results of phylogenetic analyses (Figure 5 and Figure 6).

As results, populations from three drainages show broadly consistent pattern of variation with regard to morphometric and meristic characters as well as the molecular characters. This pattern of variations in addition is supported by the ABGD analysis.

Discussion

Geographic Differentiation of Morphological Characters

The causes of observed phenotypic differences between populations are often difficult to explain. In general, changes in morphology are under the control of environmental conditions or of genetic background, or a combination of both (Scheiner, 1993; Svanbäck and Eklöv, 2006).

However, analysis of geographic variation of the morphometric and meristic characters provides more insights into the specific patterns of differentiation among the isolated populations. Based on the results obtained from the present study, the morphometric characters are considered to be suitable for discrimination of the populations of this complex group within the Iranian drainage systems. This finding is also consistent with the previous studies on the other teleosts (Sharp *et al.*, 1978).

The following characters were achieved to show high geographical differentiation among the populations from the three drainages; preanal distance, maximum body depth, depth of anal fin, head length, preorbital distance, inter orbital distance and eye diameter. These characters are often related to the head parts. It can therefore be assumed that the dimensions of the head probably represent a significant taxonomic character to differentiate the populations from the three drainages. Considering the meristic characters, the numbers of gill rakers, dorsal fin rays and of the lateral line series scales contributed slightly in differentiation of the populations from the three drainages. More likely, the observed inter-drainage morphological differentiation is resulted by geographical isolation (as vicariance). Because the current populations in Iran, have mainly been distributed in the Zagros and Alburz mountains regions. In addition, it is well known that these regions have played important role in speciation of freshwater fishes and reptiles (see below).

We assumed that the observed differentiation are not related to the environmental conditions in different habitats because all the collected populations occurred in the habitats with relatively similar environmental conditions i.e., cool river system with rapid currents and well-oxygenated water and elevation >1400 m asl (field works data of the authors). This assumption can be also supported by the fact that the morphological and molecular differentiations show consistent pattern of variation.

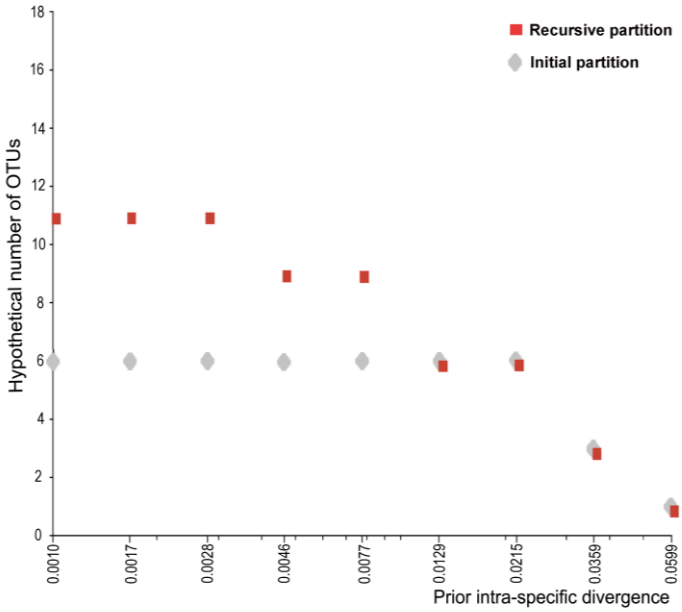

Figure 6. Automatic partition of the studied sequence data set as resulted from Automatic Barcode Gap Discovery (ABGD) analysis. The number of groups inside the partitions (primary and recursive) is reported as a function of the prior limit between intra- and interspecies divergence.

Implication of the Population Status

As mentioned above, the phenotypic and genetic differentiations that achieved for the studied populations support the hypothesis that the observed morphological differentiation among the three drainages has probably resulted by the genetic divergence because of the geographic speciation.

The studied specimens from the Southern Caspian Sea drainage formed two clusters. Cluster I comprises the specimens from Talar (M70, M71, M75), Tajan (M62, M63, M74, M628), and Babolrud (M68, M73, M682, M107) Rivers. All these rivers are located at the northeastern part of the Southern Caspian Sea drainage (Figure 1 and Figure 5). Cluster II contains individuals from Abhar (M76), Divandarreh (M66-M67), and Idaghamush (M72) Rivers. These rivers belong to the western part of the drainage (Figure 1 and Figure 5). However, this clustering was not supported by high bootstrap values. Therefore, intra-specific genetic divergence is likely for the populations from this drainage. This finding is also supported by the ABGD analysis.

Almaça (1983) has recognized two subspecies from Iranian drainages; i.e. *Barbus lacerta lacerta* from the Tigris-Karoun basin and *Barbus lacerta cyri* from the southern Caspian Sea drainage. The morphological and phylogenetic analyses in the present study confirm this finding. The populations from Tigris-Karoun drainage display slightly more numbers of gill rakers i.e., 9.07 (±075) vs. 7.27 (±0.87) in Orumiyeh and 8.25 (±0.86) in South Caspian Sea drainage (Table 6).

Berg (1948–1949) reported the South Caspian Sea drainage specimens as *B. lacerta cyri.* The type locality of this subspecies is from Kura River (valid name is now *Barbus cyri*). Another valid species is *B. armenicus* Kamenskii, 1899. Its type locality is Kars Çayı and Çıldır Lake (Aras River drainage, Turkey). It differs from *B. cyri* by more ossified last unbranched dorsal fin ray, more fleshy lower lip and developed both lateral and middle lopes (Turan, unpublished data). We examined the specimens from South Caspian Sea. They are similar to *B. cyri* regarding of the last unbranched dorsal fin ray, and both lateral and middle lopes. Therefore and because the specimens from the drainage of South Caspian Sea clustered with *B. cyri* from Kura River, we considered populations from this basin as *B. cyri*.

The populations from Orumiyeh drainage have been collected from the south part of the basin, and display relatively a short and deep head. In addition, the lateral line series scales show relatively higher range than those populations from the South Caspian Sea drainage (49-84 in Orumiyeh vs. 51-74 in South Caspian Sea). Saadati (1977) considered the populations from this basin as distinct taxa. Our study supports this suggestion as the lateral line series scales are significantly higher in Orumiyeh drainage i.e. 70.03 (±6.01) vs. 60.43 (±5.23) in South Caspian Sea drainage. Accordingly, the examined specimens from the Orumyieh basin are suggested as *Barbus* sp. However, it seems that the populations from the north parts of this basin display a different form particularly in having longer and flattened head than those from south part (shorter and deeper head). Therefore, those populations from the north part of the Orumyieh basin may be distinct taxa, which should be examined with more materials from Orumiyeh, and it should be compared with *Barbus lacerta* and *B. cyri.*

Based on the analysis of the meristic characters (numbers of lateral line series scales and gill rakers), the examined specimens from Tigris drainage in Turkey, are close to the Iranian Tigris–Karoun drainage (Table 6).

The type locality of *B. lacerta* is Kueik River (very closed to Euphrates River). Therefore and because of the close relations of the Iranian Tigris-Karoun specimens with those from Tigris- Euphrates River basin, the Tigris-Karoun populations can be accepted as *B. lacerta*.

As results, the distinctness of the populations from the three isolated drainages has been supported from both the morphological and molecular evidences. The observed differences however are supported when ABGD analysis was applied.

Geologic History, Fragmentation of Populations and Biogeographic Routes

It is well known that biogeography and taxonomic identity of this complex has been the subject of debate for decades (e.g., Almaca, 1981; Esmaeili *et al.*, 2010; Coad, 2013). Prior to date, the most studies on this species complex were limited to the morphological data and geographical distribution in local area (Valiallahi, 2006; Karaman, 1971; Berg, 1948-1949; Saadati, 1977). However, few studies have recently applied molecular evidence (e.g., Tsigenopoulos *et al.*, 2003; Tsigenopoulos and Berrebi, 2002), although they did not focus on *B. lacerta*.

As mentioned above and according to both morphological and molecular characters, both *Barbus cyri* and *B. lacerta* are known to present within the Iranian. The recent studies have suggested that active geological history of the Zagros Mountains has considerably influenced diversification of the animals in the studied region especially reptiles and freshwater fishes (Rastegar-Pouyani and Nilson, 2002; Hrbek *et al.*, 2006; Teimori *et al.*, 2012). It is particularly true in the case of freshwater fishes because of their especial pattern of distribution, in which geologic divergence can act as a major physical barrier and produce several allopatric populations.

Here we have provided a brief overview on the geological history of three drainages to discuss the possible role of geological history on isolation and diversification of the studied populations.

It is well known that Iran is a geologic active region (Dercourt *et al.*, 1986; Allen *et al.*, 2004), and its geological history has led to rapid isolations of multiple areas (Sborschchikov *et al.*, 1981; Dercourt *et al.*, 1986; Hatzfeld *et al.*, 2010). These events have probably provided an excellent condition for the speciation of different animal taxa (Macey *et al.*, 1998; Rastegar-Pouyani and Nilson, 2002; Hrbek *et al.*, 2006).

The mountain systems of the Iranian plateau were formed on Pre-Eocene plate boundaries by the Indian and Arabian collision (Abdrakhmatov *et al.*, 1996). In the beginning of the Pliocene (5 MYA), the Arabian plate accelerated separation from Africa (Girdler, 1984) and mountain building on the northern and southern margins of the Iranian plateau occurred and finally the mountain belts surrounded the north (Alburz) as well as the west and southwest (Zagros) of the Iranian plateau, where the populations of this species complex are currently distributed.

As a biogeographic point of view, the *Barbus* fauna of the studied drainages has been influenced by different zoogeographic regions (Saadati, 1977). It has been suggested that Anatolia has received its fauna from Europe after colonizing during the Oligocene, and then palearctic elements have migrated to the southwest Asia through Anatolia (Almaça, 1983, 1986). Moreover, the closing of eastern connection of Paratethys during the late Miocene (10 MYA), has triggered an exchange of *Barbus* elements between the Iranian plateau and Anatolia, so that this event facilitated dispersion of *Barbus* population directly from Anatolia to the Iranian drainage systems.

Because of the present–day geographical feature of Tigris drainages in Turkey, Iraq and Iran and possible inter-connection, therefore it is probable that Tigris-Karoun drainage in Iran has directly received its populations from the southwest Asia. We assume that the population of Tigris-Karoun drainage is probably different from those of Orumyieh and South Caspian Sea. This assumption is also supported by the fact that the individuals of Tigris-Karoun drainage in Iran showed the highest differentiation with regard to the morphological and molecular characters than two others (Figure 4 and Figure 5).

Based on a total-evidence approach including geologic history of the region (Sborschchikov *et al.*, 1981; Dercourt *et al.*, 1986; Hatzfeld *et al.*, 2010), present biogeographic distribution of the populations and the morphological as well as molecular characters, we conclude that fragmentation of the geographic unites owing to vicariance events since approximately 5–10 MYA are probably the major factors that influenced diversification of this species complex within Iranian the drainages.

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References

Abdoli, A. 2000. The Inland Water Fishes of Iran. Iranian Museum of Nature and Wildlife. Tehran, 378 pp.

Abdrakhmatov, K.Y., Aldazhanov, S.A., Hager, B.H., Hamburger, M.W., Herring, T.A., Kalabaev, K.B., Makarov, V.I., Molnar, P., Panasyuk, S.V., Prilepin, M.T., Reilinger, R.E., Sadybakasov, I.S., Souter, B.J., Trapeznikov, Y.A., Tsurkov, Y.V. and Zubovich, A.V. 1996. Relatively recent construction of the Tien Shan inferred from GPS measurements of present-day crustal deformation rates. Nature, 384: 450–453. doi:10.1038/384450a0.

Allen, M., Jackson, J. and Walker, R. 2004. Late Cenozoic reorganization of the Arabia-Eurasia collision and the comparision of short-term and long term deformation rates. Tectonics, 23:3–16. doi:10.1029/2003TC001530, 2004.

Almaça, C. 1981. La collection de *Barbus* d'Europe du Muséum national d'Histoire naturelle (Cyprinidae, Pisces). Bulletin du Muséum national d'Histoire naturelle, Paris, 4^e série, 3, section A, numéro 1: 277–307.

Almaça, C. 1983. Evolutionary, biogeographical, and taxonomical remarks on Mesopotamian species of *Barbus s.s.* Arquivos do Museu Bocage, 4: 63–78.

Almaça, C. 1986. On some *Barbus* species from Western Asia (Cyprinidae, Pisces). Annalen des Naturhistorischen Museums in Wien, 87: 5–30.

Berg, L.S. 1949. Presnovodnye ryby Irana i sopredel'nykh stran [Freshwater fishes of Iran and adjacent countries]. Trudy Zoologicheskogo Instituta Akademii Nauk SSSR, 8: 783–858.

Berg, L.S. 1948–1949. Freshwater fishes of the USSR and adjacent countries. Israel Program for Scientific Translations. Jerusalem, 1962–1965.

Berrebi, P., Kottelat, M., Skelton, P. and Ráb, P. 1996. Systematics of *Barbus*: state of the art and heuristic comments. Folia Zoologica, 45: 5–12.

Bianco, P.G. 1995. Revision of the Italian *Barbus* species (Cypriniformes: Cyprinidae). Ichthyological Exploration of Freshwaters, 6: 305–324.

Bianco, P.G. and Banarescu, P. 1982. A contribution to the knowledge of the Cyprinidae of Iran (Pisces, Cypriniformes). Cybium, 6: 75–96.

Coad, B.W. 1987. Zoogeography of the freshwater fishes of Iran. In: F. Krupp, W. Schneider and R. Kinzelbach (Eds.), Weisbaden Proceedings of the Symposium on the Fauna and Zoogeography of the Middle East. Beihefte zum. TAVO A, 28: 213-228.

Coad, B.W. 1998. Systematic biodiversity in the freshwater fishes of Iran. Italian Journal of Zoology, 65: 101–108. doi: 10.1080/11250009809386802.

Coad, B.W. 2013. Freshwater fishes of Iran. Available from http://www.briancoad.com, Accessed 20 Jan 2013.

Coad, B.W. and Vilenkin, B.Y. 2004. Co-occurrence and zoogeography of the freshwater fishes of Iran. Zoology in the Middle East, 31: 53–61. doi:10.1080/09397140.2004.10638022.

Dercourt, J., Zonenshain, L.P., Ricou, L.E., Kazmin, V.G., Pichon, L.E., Knipper, A.L., Grandjacquet, C., Sbortshikov, I.M., Geyssant, J., Lepvrier, C., Pechersky, D.H., Boulin, J., Sibuet, J.C., Savostin, L.A., Sorokhtin, O., Westphal, M., Bazhenov, M.L., Lauer, J.P., Biju-Duval, B., Pichon, L.E. and Monin, A.S. 1986. Geological evolution of the Tethys belt from the Atlantic. Tectonophysics, 123: 241–315. doi: 10.1016/0040-1951(86)90199-X.

Durand, J.D., Tsigenopoulos, C.S., Ünlü, E. and Berrebi, P. 2002. Phylogeny and biogeography of the family Cyprinidae in the Middle East inferred from cytochrome *b* DNA – evolutionary significance of this region. Molecular Phylogenetics and Evolution 22: 91–100. doi: 10.1006/mpev.2001.1040.

Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research, 32: 1792–97. doi: 10.1093/nar/gkh340.

Esmaeili, H.R., Coad, B.W., Gholamifard, A., Nazari, N. and Teimori, A. 2010. Annotated checklist of the freshwater fishes of Iran. Zoosystematica Rossica, 19: 361–386.

Girdler, R.W. 1984. The evolution of the Gulf of Aden and Red Sea in space and time. Deep-Sea Research, 31: 747–762. doi: 10.1016/0198-0149(84)90039-6.

Goldstein, P.Z. and DeSalle, R. 2011. Integrating DNA barcode data and taxonomic practice: Determination, discovery, and description. Bioessays, 33: 135–147. doi:10.1002/bies.201000036;

Hammer, Ø., Harper, D.A.T. and Ryan, P.D. 2001. PAleontological STatistics Software Package For Education And Data Analysis. Palaeontologia Electronica. http://palaeo-electronica.org.

Hatzfeld, D., Authemayou, C., Vanderbeek, P., Bellier, O., Lave,´ J., Oveisi, B., Tatar, M., Tavakoli, F., Walpersdorf, A. and Yamini-Fard, F. 2010. The kinematics of the Zagros Mountains (Iran). Geological Society of London, 330: 19–42. doi:10.1144/SP330.3.

Hebert, P.D.N., Cywinska, A.L., Ball, S.R. and deWaard, J. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society, 270(B): 313–321. doi:10.1098/rspb.2002.2218.

Holcik, J. 1989. The Freshwater Fishes of Europe. General Introduction to Fishes (Acipenseriformes). AULA-Verlag, 469 pp.

Hrbek, T., Keivany, Y. and Coad, B.W. 2006. New species of *Aphanius* (Teleostei, Cyprinodontidae) from Isfahan Province of Iran and a reanalysis of other Iranian species. Copeia, 2: 244–255. doi: 10.1643/0045-8511(2006)6[244:NSOATC]2.0.CO;2.

Huelsenbeck, J.P. and Ranala, B. 2004. Frequentist properties of Bayesian posterior probabilities of phylogenetic trees under simple and complex substitution models. Systematic Biology, 53: 904–913. doi: 10.1080/10635150490522629.

Karaman, M.S. 1971. Süßwasserfische der Türkei. Revision der Barben Europas, Vorderasiens und Nordafrikas. Mitteilungen aus dem hamburgischen Zoologischen Museum und Institut, 67: 175–254.

Kiabi, B.H., Abdoli, A. and Naderi, M. 1999. Status of the fish fauna in the South Caspian drainage of Iran. Zoology In The Middle East, 18: 57–65.

Kottelat, M. and Freyhof, J. 2007. Handbook of European freshwater fishes. Publications Kottelat, Cornol, Switzerland, 646 pp.

Lahnsteiner, F. and Jagsch, A. 2005. Change in phenotype and genotype of Austrian *Salmo trutta* populations during the last century. Environmental Biology of Fish, 74: 51–65. doi: 10.1007/s10641-005-4420-9.

Macey, JR., Schulte, J.A., Ananjeva, N.B., Larson, A., Rastegar-Pouyani, N., Shammakov, S.M. and Papenfuss, T.J. 1998. Phylogenetic relationships among agamid lizards of the *Laudakia caucasia* species group: Testing hypotheses of biogeographic fragmentation and an area cladogram for the Iranian Plateau. Phylogenetics and Evolution, 10: 118–131.

Machordom, A. and Doadrio, I. 2001. Evidence of a Cenozoic Betic-Kabilian connection based on freshwater fish phylogeography (*Luciobarbus*, Cyprinidae). Phylogenetics and Evolution, 18: 252–263. doi: 10.1006/mpev.2000.0876.

Motamedi, M. 2010. Taxonomy of *Barbus lacerta* complex within the Iranian drainage systems; Evidence by morphological and molecular data. MSc thesis. Kerman: Shahid-Bahonar-University of Kerman.

PASW, 20.00. SPSS Inc, 2012. IL: SPSS, Inc. Chicago.

Perea, S., Böhme, M., Zupančič, P., Freyhof, J., Šanda, R., Özuluğ, M., Abdoli, A. and Doadrio, I. 2010. Phylogenetic relationships and biogeographical patterns in Circum-Mediterranean subfamily Leuciscinae (Teleostei, Cyprinidae) inferred from both mitochondrial and nuclear data. BMC Evolutionary Biology, 10: 265. doi:10.1186/1471-2148-10-265.

Puillandre, N., Lambert, A., Brouillet, S. and Achaz, G. 2012. ABGD, Automatic Barcode Gap Discovery for primary species delimitation. Molecular Ecology, 21: 1864–1877. doi: 10.1111/j.1365-294X.2011.05239.x.

Rastegar–Pouyani, N. and Nilson, G. 2002. Taxonomy and Biogeography of the Iranian Species of *Laudakia* (Sauria: Agamidae). Zoology in the Middle East, 26: 93–122. doi:10.1080/09397140.2002.10637926.

Ronquist, F. and Huelsenbeck, J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics, 19: 1572–1574. doi: 10.1093/bioinformatics/btg180.

Saadati, M.A.G. 1977. Taxonomy and distribution of the freshwater fishes of Iran. Master thesis, Colorado State University, Fort Collins.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: a Laboratory Manual, 2nd Edn., Cold Spring Harbor, Colad Spring Harbor Laboratory Press. New York, 626 pp.

Sborschchikov, I.M., Savostin, L.A. and Zonenshan, L.P. 1981. Present plate tectonics between Turkey and Tibet. Tectonophysics, 79: 45–37. doi: 10.1016/0040-1951(81)90232-8.

Scheiner, S.M. 1993 Genetics and evolution of phenotypic plasticity. Annual Reviews in Ecology and Systematics, 24: 35–68. doi: 10.1146/annurev.es.24.110193.000343.

Sharp, J.C., Able, K.W. and Legget, W.C. 1978. Utility of meristic and morphometric characters for identification of cepelin (*Mallotus villosus*) stocks in Canadian Atlantic waters. Journal of the Fisheries Research Board of Canada, 35: 124–130. doi:10.1139/f78-016.

Stamatakis, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics, 22: 2688–2690. doi: 10.1093/bioinformatics/btl446;

Svanbäck, R. and Eklöv, P. 2006. Genetic variation and phenotypic plasticity: causes of morphological and dietary variation in Eurasian perch. Evolutionary Ecology Research, 8: 37–49.

Teimori, A., Esmaeili, H.R., Gholami, Z., Zarei, N. and Reichenbacher, B. 2012. *Aphanius arakensis*, a new species of tooth-carp (Actinopterygii, Cyprinodontidae) from the endorheic Namak Lake basin in Iran. ZooKeys, 215: 55–76. doi: 10.3897/zookeys.215.1731.

Tsigenopoulos, C.S. and Berrebi, P. 2000. Molecular phylogeny of North Mediterranean freshwater barbs (genus *Barbus*: Cyprinidae) inferred from cytochrome b sequences: biogeographic and systematic implications. Molecular Phylogenetics and Evolution, 14: 165–179. doi: 10.1006/mpev.1999.0702.

Tsigenopoulos, C.S., Durand, J.D., UÅNnlü, E. and Berrebi, P. 2003. Rapid radiation of the Mediterranean *Luciobarbus* species (Cyprinidae) after the Messinian salinity crisis of the Mediterranean Sea, inferred from mitochondrial phylogenetic analysis. Biological Journal of the Linnaean Society, 80: 207–222.

Valiallahi, J. 2006. Identification of *Barbus plebjus* (Bonaparte, 1832) a valid species of cyprinid fish from Iran. Iranian Journal of Biology, 19: 109–116.

Veasey, E.A., Schammass, E.A., Vencovsky, R., Martins, P.S. and Bandel, G. 2001. Germplasm characterization of Sesbania accessions based on multivariate analyses. Genetic Resources and Crop Evolution, 4879–4890. doi: 10.1023/A:1011238320630.