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Mitochondrial Genetic Differentiation of Spirlin (Actinopterigii: Cyprinidae) in the South Caspian Sea basin of Iran

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Abstract

Background: Knowledge about *Alburnoides* remains lacking relative to many other species, resulting in a lack of a systematic position and taxonomic diagnosis. Basic biological information for *Alburnoides* has been constructed, and it is necessary to understand further and obtain more information about this species. Its phylogenetic relationships are still debated and no molecular data have been used to study this taxon in Iran. A holistic approach for genetic methods was adopted to analyze possible spirlin population differences at selected centers in the south Caspian Sea basin of Iran.

Methods: The phylogenetic relationships were determined based on 774 base pairs of the mitochondrial cytochrome b gene of 32 specimens of spirlin from nine locations in the south Caspian Sea drainage basin of Iran. The nucleotide sequences were subjected to phylogenetic analysis using the neighbor-joining, maximum parsimony, maximum likelihood, and Bayesian methods.

Results: The mitochondrial gene tree largely supports the existence of three major clades. The western populations (clade I) may be considered as *Alburnoides eichwaldii*, whereas the Talar river populations (clade II) are represented as *Alburnoides* sp.1 and the eastern populations (clade III) may be distinct taxa of *Alburnoides* sp.2.

Conclusion: This molecular evidence supports the hypothesis that *A. bipunctatus* does not exist in the south Caspian Sea basin of Iran, and that the western and eastern populations are distinct taxa.

Keywords: *Alburnoides*, spirlin, cytochrome b, phylogenetic relationships, Caspian Sea, Iran

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Introduction

Different kinds of freshwater fishes of the Cyprinidae genus, commonly known as spiralin, are widely distributed in the freshwaters of Europe, the Caucasus, Asia Minor, and Central Asia, where it is represented by 17 species.¹⁻⁴ Spirilins are small cyprinid fish generally found in shallow areas with gravel, stones, and well oxygenated clear water.

This taxon has long been considered as a complex species with great diversity in Iran. Six species were recently recognized in the Iranian basins of the Caspian Sea, Kavir, Namak, Tigris, Kor, and Lake Orumiyyeh, and more are expected to be discovered.^{3,5,6} The spread of spiralin across Iran has important ecological, zoogeographic, and aquacultural significance.⁷

Previous molecular work on *Alburnoides*, mostly European species, has been based on allozyme, cytochrome b, 16s rDNA, and mitochondrial control region data.^{8,9} Morphologic and genetic markers provide different but complementary information about population structure and have been widely used in population differentiation studies.¹⁰⁻¹³ Molecular tool approaches are powerful techniques to investigate geographic variation of stocks. Among the molecular studies, mitochondrial DNA has proved to be useful in the study of population diversity and provides information on fish characteristics.¹⁴⁻¹⁶

Alburnoides belongs to a number of poorly studied species with a deficiency in molecular analyses and no consensus among ichthyologists concerning its taxonomic position, so knowledge and information regarding this species is largely missing. The situation is more complicated in the Caspian Sea basin, and there are still debates on the taxonomic position of the spiralin found in the south Caspian Sea basin. *Alburnoides bipunctatus*

eichwaldii was the name used for most populations across the south Caspian Sea basin of Iran. Bogutskaya and Coad³ resurrected *A. eichwaldii*, and it is present at least in the western part of the Caspian Sea basin, west of Sefidrud. There is another nominal subspecies in the Aras River drainage region of Armenia (Caspian Sea basin), ie, *A. bipunctatus armeniensis*, Dadikyan, 1972.¹⁷ From the rivers of Arpa, Vorotan, Vedi, Marmarik, and Kasakh as well as their tributaries, which is now regarded as a synonym of *A. eichwaldii*.³ However, the status of the eastern populations is not clear, and these may be considered to be a distinct species. Hence, knowledge and information regarding the south Caspian spiralin remains limited. These taxonomic problems persist because most of the earlier investigations were solely based on classical techniques. The use of molecular data to infer phylogenetic trees are used to complement morphological data in order to study relationships in detail. This is the first documentation of the systematic position and phylogenetic relationship between the different populations of *Alburnoides* integrating molecular techniques (nucleotide sequence of the mitochondrial cytochrome b gene) in the Caspian Sea basin of Iran.

Materials and Methods

Sampling

This study was based on 32 specimens of spiralin collected from nine locations in the south Caspian Sea drainage basin of Iran using an electrofishing device (Table 1 and Fig. 1).

DNA extraction and sequencing

In the laboratory, muscle tissues were dissected immediately and stored at -20°C prior to total

Table 1. Sampling locations of spiralin in Iran.

	Code	Collecting site	Longitude E	Latitude N
1	Cr	Sefidrud-Chenar Rudkhan	49° 27' 26.1"	31° 04' 42.4"
2	Do	Sefidrud-Dorudkhan	49° 24' 56.9"	37° 04' 3.6"
3	Gz	Gorganrud-Zaringol	54° 57' 27"	36° 51' 46"
4	Ks	Talar-Kesselian	52° 59' 46"	36° 13' 33"
5	Mp	Gorganrud-Madarsoo	55° 50' 47.6"	37° 23' 3.2"
6	Sd	Sefidrud-Ramsar-Safarud	50° 37' 56.8"	36° 54' 52.6"
7	Tu	Talar-Tuji	52° 53' 14.5"	36° 22' 2.5"
8	Vt	Sefidrud-Tonekabon-Valmrud	50° 50' 59.1"	36° 44' 36"
9	Zl	Sefidrud-Zeleki	49° 37' 59.4"	37° 01' 9.6"

Abbreviations: Sd, Safarud; Zl, Zeleki; Do, Dorudkhan; Cr, Chenarrudkhan; Vt, Valamrud; Tu, Tuji; Ks, Kesselian; Gz, Gorganrud; Mp, Madarsoo.

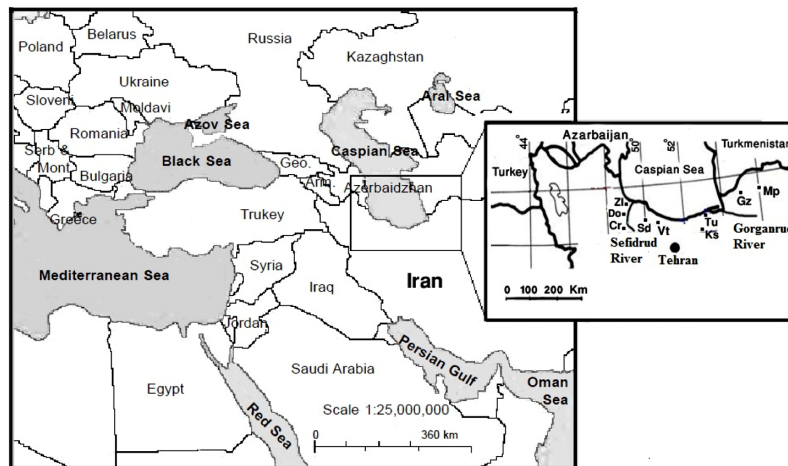


Figure 1. Map of sampling locations in the south Caspian Sea drainage basin of Iran.

Abbreviations: Sd, Safarud; Zl, Zeleki; Do, Dorudkhan; Cr, Chenarrudkhan; Vt, Valamrud; Tu, Tuji; Ks, Kesselian; Gz, Gorganrud; Mp, Madarsoo.

DNA extraction. DNA was extracted from the tissue using the cetyl-trimethylammonium bromide method.¹⁸ A fragment of cytochrome b was sequenced using two primer combinations that amplify a region of 774 base pairs starting from the 5' terminus of the mitochondrial cytochrome b primers in the flanking (GluDG.L TGA^{CTT}GAA^{RAAC}CAYCGTT) and threonine (cb6b.H GGA^{ATT}CACCTCTCC) regions.¹⁹ Polymerase chain reaction products were cleaned for sequencing and aligned using Mega 4.²⁰ Alignment of cytochrome b was based on the inferred amino acid sequence. Our data set was complemented by cytochrome b sequences of four *A. bipunctatus* (GenBank accession AF090740, HM560059, Y10445, and AF090741). The sequences generated in this study for each gene have been submitted to GenBank under accession numbers (HQ658865–HQ658893, Table 2).

Phylogenetic and Bayesian analyses

The nucleotide sequences obtained were subjected to phylogenetic analysis by the neighbor-joining,²¹ maximum parsimony, and maximum likelihood methods using the PAUP 4.0b10 program.²² The aligned sequences were also estimated by Bayesian inference using MrBayes 3.1.2.²³ The choice of model for the Bayesian inference and maximum likelihood method was determined based on the Akaike information criterion as implemented in the Model test²⁴ by Aliabadian et al.²⁵ For maximum likelihood purposes, the estimated models were used in a subsequent maximum likelihood heuristic tree search with 10 random

addition sequence replicates, using tree bisection and reconnection branch swapping. To assess the nodal support for each branch, 100 and 1000 bootstrap replicates were run under maximum likelihood and maximum parsimony, respectively, with parameters estimated automatically. The choice of model for the Bayesian analyses was determined based on an Akaike information criterion as implemented in the Model test. With four Metropolis-coupled MCMC chains, incremental heating temperatures of 0.1 °C were run for 20 million generations and sampled every 100 generations. The first 10 million generations of each run, well after the chain reached apparent stationarity, were discarded as burn-in. The resulting 50% majority rule consensus tree was considered for the hypothesis.

The Kimura-2 parameter distance was used to calculate the genetic distances for cytochrome b to quantify sequence divergences between individuals.²⁰ We compared alternative phylogenetic hypotheses using the Shimodaira-Hasegawa test,²⁶ as implemented in RAxML version 7.0.3.²⁷ The tested topologies were obtained, enforcing the monophyly of 10 selected populations in the maximum likelihood searches for RAxML.²⁷

Results

Sequence characteristics and phylogenetic tree

A total of 32 partial cytochrome b sequences from nine locations in the Caspian Sea basin were analyzed. Among the 774 base pairs of the mitochondrial

**Table 2.** Tissue samples and GenBank accession number for cytochrome b.

No.	Accession (cyt b)	Code	Code locality
1	HQ658865	2392	Ks
2	HQ658866	760	Ks
3	HQ658867	759	Ks
4	HQ658868	763	Ks
5	HQ658869	1602	Sd
6	HQ658870	1597	Sd
7	HQ658871	1591	Sd
8	HQ658872	1608	Sd
9	HQ658873	1587	Sd
10	HQ658874	634	Tu
11	HQ658875	635	Tu
12	HQ658876	1252	Vt
13	HQ658877	1253	Vt
14	HQ658878	1255	Vt
15	HQ658879	1254	Vt
16	HQ658880	1528	Cr
17	HQ658881	1535	Cr
18	HQ658882	1534	Cr
19	HQ658883	1467	Do
20	HQ658884	1459	Do
21	HQ658885	1351	ZI
22	HQ658886	1342	ZI
23	HQ658887	1330	ZI
24	HQ6588863	1397	ZI
25	HQ658888	1187	Mp
26	HQ658889	1179	Mp
27	HQ658890	1196	Mp
28	HQ658891	704	Gz
29	HQ658892	703	Gz
30	HQ658893	702	Gz
31	HQ658894	701	Gz
32	HQ658864	710	Gz

Abbreviations: cyt b, cytochrome b; Sd, Safarud; ZI, Zeleki; Do, Dorudkhan; Cr, Chenarrudkhan; Vt, Valamrud; Tu, Tuji; Ks, Kesselian; Gz, Gorganrud; Mp, Madarsoo.

cytochrome b gene, 130 base pair positions were variable and 92 base pairs were phylogenetically informative sites. A statistical summary of the maximum parsimony analysis for cytochrome b data sets and model selected by the Model test for the cytochrome b gene is shown in Table 3.

The neighbor-joining and maximum parsimony analyses produced a tree topology similar to maximum likelihood and Bayesian inference. Results of the three character state methods, ie, maximum parsimony, maximum likelihood and Bayesian analyses, represent the three major clades, namely, clade I, clade II, and clade III, with strong posterior probability support in Bayesian analysis, bootstrap values in maximum likelihood, and bootstrap values

Table 3. Results of maximum parsimony and maximum likelihood analysis.

Information of MP and ML analysis	Cyt b
Number of parsimony informative characters	92/774
Number of conserved characters	644/744
Number of variable sites	130/774
Number of most parsimonious trees obtained	161/267
Consistency index	0.747
Retention index	0.939
Base frequencies	A = 0.279 C = 0.2828 G = 0.1635 T = 0.2740
Model selected by model test	GTR + G
Assumed proportion of invariable sites (pinv)	0.49
Gamma distribution shape parameter (a)	0.77

Abbreviations: cyt b, cytochrome b; MP, maximum parsimony; ML, maximum likelihood.

in maximum parsimony; therefore, for economy of space, only the resulting Bayesian trees are presented (Fig. 2). Clade I included five populations from Safarud, Zeleki, Dorudkhan, Chenarrudkhan, and Valamrud, with values of 100/100/99 posterior probability for Bayesian analyses, maximum likelihood, and maximum parsimony bootstrap, respectively. They mostly belong to the western (Sefidrud) drainage region of the south Caspian Sea basin. Clade II (Tuji and Kesselian) represented populations with values of 100/84/98 in the central (Talar) drainage region of the south Caspian Sea basin. Populations including Gorganrud and Madarsoo in the southeastern Caspian Sea (Gorganrud drainage basin) make the distinct clade III, with 100 bootstrap values using all methods. There was a sister-group relationship between Kesselian and Tuji (PP = 100/84/98) in clade II and a sister-group relationship between Madarsoo and Gorganrud (PP = 100/100/100) in clade III (Fig. 2).

Evolutionary distance of species

In general, the pairwise distance in the Caspian spirulin was 0.34%. Intraspecific (K2P) distances ranged from 0% to 0.3% (Table 4). The largest amount of mean intraspecific divergence was displayed in the population of Chenarrudkhan (Table 4). Interspecific (K2P) distances ranged from 0% to 0.64% (Table 5).

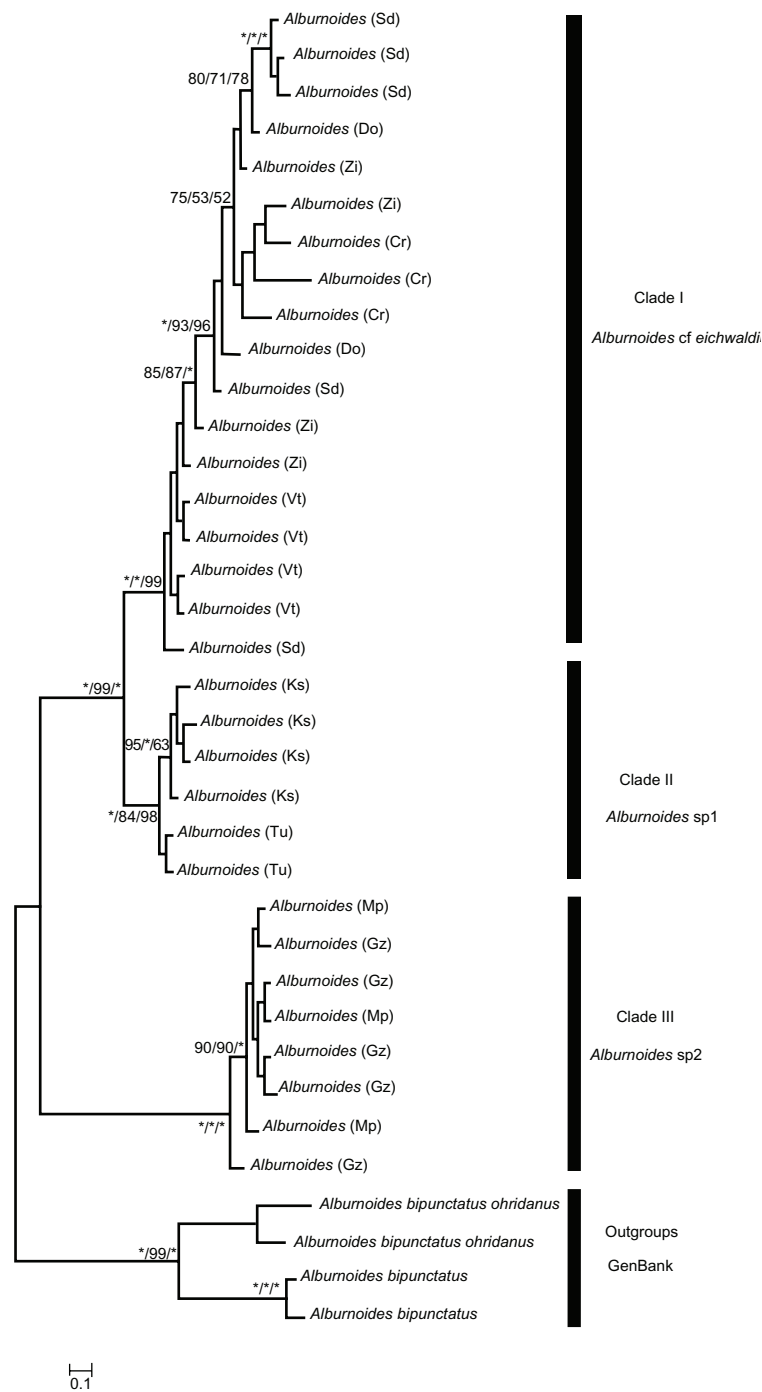


Figure 2 Bayesian consensus tree resulting from analysis of 774 base pairs of cytochrome b data in the south Caspian Sea basin of Iran only. **Notes:** Values at branches indicate, in sequence, posterior probability, maximum likelihood, and maximum parsimony bootstrap (all >0.50/50%). *Posterior probability or bootstrap value of 1.00/100%. **Abbreviations:** Sd, Safarud; Zl, Zeleki; Do, Dorudkhan; Cr, Chenarrudkhan; Vt, Valamrud; Tu, Tuji; Ks, Kesselian; Gz, Gorganrud; Mp, Madarsoo.

The largest amount of mean interspecific divergence was displayed between the Madarsoo and the Kesselian and Tuji populations (0.71%), as shown in Table 5, and the greatest amount of mean interspecific divergence among clades was displayed between clade I and clade III (Table 6).

Shimodaira-Hasegawa tests

Our results are reinforced by the Shimodaira-Hasegawa test showing the reciprocal monophyly of all selected populations, except for those from Safarud, Zeleki, and Dorudkhan. Only the maximum likelihood trees resulting from constraint searches obtained



Table 4. Intraspecific pairwise distances of mitochondrial cytochrome b gene based on Kimura-2 parameter methods.

Population	K2P%
Zi	0.008
Sd	0.006
Vt	0.000
Cr	0.009
Do	0.003
Ks	0.001
Tu	0.000
Mp	0.001
Gz	0.003
Outgroups	0.031

Abbreviations: cyt b, cytochrome b; Sd, Safarud; Zi, Zeleki; Do, Dorudkhan; Cr, Chenarrudkhan; Vt, Valamrud; Tu, Tuji; Ks, Kesselian; Gz, Gorganrud; Mp, Madarsoo.

enforcing the monophyly of the populations from Safarud, Zeleki, Dorudkhan were significantly worse than the tree obtained from the non-constraint search for a cytochrome b data set. Results of Shimodaira-Hasegawa tests performed with RAxML and the difference in tree likelihood compared with the best tree exactly were similar and confirmed the results of the Bayesian maximum likelihood and maximum parsimony trees concerning the monophyly of all three clades and non-monophyly of the three populations from Safarud, Zeleki, and Dorudkhan (Table 7).

Discussion

The aim of this study was to obtain an overview of the relationships between the *Alburnoides* (Bloch, 1782) populations along the Caspian Sea. Molecular analysis of the cytochrome b nucleotide sequence data indicates that the *Alburnoides* populations should be reviewed, and that *A. bipunctatus* is not a valid name for the Iranian populations (Fig. 2), because outgroups in this study (*A. bipunctatus* and *A. ohridanus*) of the European *Alburnoides* comprise a distinct clade (Fig. 2).

Table 5. Interspecific pairwise distances of mitochondrial cytochrome b gene based on Kimura-2 parameter methods among different populations.

Clades	Clade I	Clade II	Clade II	Outgroups
Clade I	0			
Clade II	0.019			
Clade III	0.066	0.062		
Outgroups	0.076	0.073	0.088	0

The present mitochondrial DNA data suggest that the southwestern populations in the Sefidrud drainage basin (clade I) may be considered as *A. eichwaldii*, whereas the Talar river populations (clade II) might be a distinct taxon of *Alburnoides* sp1. The clade of eastern populations (Gorganrud drainage basin, clade III) namely, Madarsoo and Gorganrud, are clearly separated from that of the Talar (clade II) and Sefidrud (clade I) drainage basins, making a distinct species (*Alburnoides* sp2).

The results obtained in this research confirmed the hypothesis of Zardoya and Doadrio²⁸ and of Zehzad et al²⁹ about biogeographic distribution of the populations, showing that fragmentation of land is probably the main cause for the observed molecular differentiation within the Iranian ichthyogeographic basins, and possible explanations for divergence in populations of fish may be behavioral mechanisms and historic barriers to gene flow.

The geology of Iran suggests rapid isolation of multiple areas from one another. Extensive indentation of the Arabian plate into the Iranian plate starting 10 million years ago³⁰ caused uplifting of the Zagros mountains at the southern edge of the Iranian plate. Continued northeastern movement of the Arabian plate and a northerly movement of India resulted in additional mountain building by 5 million years ago³⁰ along the northern edge of the Iranian plateau as well as along the sutures of the Iranian, Lut, and Helmand plates, making different drainage basins. These events have affected the gene flow of different populations present in this area. Description of new *Alburnoides* taxa, including *A. namaki* (Namak basin), *A. qanati* (Kor basin), *A. petrubanarescui* (Urmia basin), *A. nicolausi* (Tigris basin), and *A. idignensis* (Tigris basin), which have already been considered as a complex *A. bipunctatus* group, revealed the distinct geomorphologic histories of these basins.

The south Caspian Sea originated as a back-arc basin at some time in the Jurassic-Paleogene interval. The interior of the south Caspian basin is relatively seismic compared with the mountain ranges around it.^{31,32} In summary, synchronous deposition and deformation of the sedimentary cover of the south Caspian basin in the last 3–5 million years is consistent with incipient subduction of its rigid basement northward under the middle Caspian region. The south Caspian region is unlikely to accommodate a major part of the Arabia-Eurasia convergence. If South Caspian-Eurasia motion

**Table 6.** Interspecific pair-wise distances of mitochondrial cytochrome b gene based on Kimura 2 parameter methods among different clades.

	Zi	Sd	Vt	Cr	Do	Ks	Tu	Mp	Gz	Outgroups
Zi	0									
Sd	0.001	0								
Vt	0.003	0.002	0							
Cr	0.000	0.001	0.005	0						
Do	0.001	0.001	0.003	0.000	0					
Ks	0.016	0.016	0.014	0.018	0.017	0				
Tu	0.015	0.015	0.013	0.017	0.016	0.000	0			
Mp	0.063	0.062	0.060	0.064	0.063	0.071	0.071	0		
Gz	0.062	0.062	0.060	0.064	0.063	0.059	0.061	0.021	0	
Outgroups	0.059	0.059	0.058	0.062	0.059	0.058	0.057	0.065	0.052	0

Abbreviations: cyt b, cytochrome b; Sd, Safarud; Zi, Zeleki; Do, Dorudkhan; Cr, Chenarrudkhan; Vt, Valamrud; Tu, Tuji; Ks, Kesselian; Gz, Gorganrud; Mp, Madarsoo.

is 5 mm per year, it could achieve the 15 km of shortening estimated in the basin fill in 3 million years.

This basin, in its land part, is elongated, extending from the Turkish border almost to the Afghan border, and only acquires some width where the Sefidrud and its tributaries penetrate the Alborz mountains in the west. The south Caspian basin underlies the southern part of the Caspian Sea, between the ranges of the eastern Greater Caucasus, Talysh, Alborz, and Kopet Dagh.

The ancient origins of the south Caspian Sea, multiple sources of species, wide latitudinal extent, uneven distribution of inflows and nutrient inputs, and low to moderate salinity have all contributed to the high

biodiversity found in the region. It is the remnant of the ancient, mainly Parathethys Sea therefore; the role of closing of the Tethys Sea on distribution and then differentiation of the current populations is not unexpected.

The greatest ichthyodiversity in the Iranian drainage basin is seen at the Caspian Sea basin, which comprises both fresh and brackish habitats, rivers, lakes, lagoons, marshes, and marine environments, and has had a long history of connection and isolation from fresh and marine waters.³² The presence of some endemic native fish, ie, *Rhodeus* sp., *Squalius orientalis*, *Rutilus caspicus*, *Vimba persa*, *Salmo caspius*, *Atherina caspia*, *Syngnathus caspius*, *Cobitis fardipaki*,³³ and further as yet undescribed

Table 7. Comparison of alternative phylogenetic hypotheses using the Shimodaira-Hasegawa test performed with RAxML.

Topology tested*	(Best ML tree)	(D) ln L	SD	Significantly worth
Best tree	-2009.86309			
Monophyly of Sd	-2027.939127	-18.076037	8.053215	Yes
Monophyly of Zi	-2115.026948	-105.16386	14.387983	Yes
Monophyly of Cr	-2011.575554	-1.712464	5.755113	No
Monophyly of Tu	-2009.863178	-0.000088	0.000447	No
Monophyly of Vt	-2009.863266	-0.000176	0.000590	No
Monophyly of Mp	2009.863347	-0.000257	0.000526	No
Monophyly of Do	-2143.220385	-133.357295	17.460694	Yes
Monophyly of Ks	-2028.157341	-18.294251	13.822117	No
Monophyly of Gz	-2028.157341	-18.294251	13.822117	No
Monophyly of clade I	2009.863347	-0.000257	0.000526	No
Monophyly of clade II	-2018.016542	-8.153452	12.624665	No
Monophyly of clade III	-2009.86327	-0.000181	0.00064	No
Monophyly of outgroup	-2009.863290	-0.000200	0.000822	No

Note: *Difference in tree likelihood compared with the best tree.

Abbreviations: cyt b, cytochrome b; Sd, Safarud; Zi, Zeleki; Do, Dorudkhan; Cr, Chenarrudkhan; Vt, Valamrud; Tu, Tuji; Ks, Kesselian; Gz, Gorganrud; Mp, Madarsoo.



species, reveal the long history of its connection and isolation from fresh and marine waters due to the geomorphology of the region.

The wide distribution of *Alburnoides* in the south Caspian Sea basin of Iran from the west (Aras River, eastern Greater Caucasus) to the east (Atrak River, Kopet Dagh), with such different habitat conditions, may be responsible for speciation of *Alburnoides* populations and formation of distinct taxa.

According to Mendel,³⁴ analyses of the mitochondrial cytochrome b gene have revealed that *A. bipunctatus* is a complex group found from France to Afghanistan, comprising five monophyletic groups, ie, I (*A. bipunctatus*, France, Czech Republic, Slovakia), II (*Alburnoides* sp., Croatia), III (*A. bipunctatus ohridanus*, adopted from GenBank database, Greece), IV (*A. bipunctatus strymonicus*, the subspecific name from GenBank database, Greece), and V (*Alburnoides* sp, Russia). Based on this evidence, various *Alburnoides* populations occurring in isolated basins of the Zagros and Elburz mountains and the intervening Iranian plateau form a monophyletic group. However, relationships between these populations are likely to approximate star-like radiation.³⁴

There is remarkably good agreement between the results obtained using Shimodaira-Hasegawa²⁶ tests and molecular trees for the different methods (Bayesian inference, maximum likelihood, and maximum parsimony, Table 6 and Fig. 2). Also, there is a remarkable match between the molecular phylogeny and genetic distances among species in this study, the largest interspecific distance belonging to the populations of Madarsoo and Gorganrud in clade III (Table 5). Also, the largest interspecific distances among tree clades were found for Clade I and Clade III and then for Clade II and Clade III (Table 6).

In conclusion, our results provide molecular data which are useful for revising the systematic position of *Alburnoides* populations in the south Caspian Sea basin. This molecular evidence supports the hypothesis that *A. bipunctatus* does not exist in the south Caspian Sea basin of Iran, and that the western and eastern populations are distinct taxa.

Author Contributions

Analysed the data: FYM. Wrote the first draft of the manuscript: FYM. Contributed to the writing of the

manuscript: HRE. All authors read and approved the final manuscript.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest. The authors report no conflicts of interest in this work.

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