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PROOF

Molecular Systematic Analysis of Shad Species (*Alosa* spp.) from Turkish Marine Waters using mtDNA Genes

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Abstract

The phylogenetic relationship among five shad species (*Alosa caspia*, *A. fallax nilotica*, *Alosa maeotica*, *Alosa immaculata*, *Alosa tanaica*) from Turkish marine waters was investigated with mitochondrial DNA polymerase chain reaction-restriction fragment length polymorphism.The six genesegments, NADH 5/6, NADH 3/4cytochrome b, COX, 16 SrRNA and D-Loop,of mtDNA amplified by PCR were digested with seven restriction enzymes, *Bsur*I, *Alu*I, *Ehe*I, *Hin6*I, *Rsa*I, *Xho*I *Bsh1236*I,respectively.When all the six genes were combined together for phylogenetic analysis, a total of 45 haplotypes were detected from the five shad species, and the average haplotype diversity and nucleotide diversity within species were 0.8809 and 0.0022 respectively. The average nucleotide diversity and nucleotide divergence among species were 0.009248 and 0.007080 respectively. The highest genetic divergence was observed between *A. caspia*and *A. maeotica* (0.013727) and the lowest between *A. immaculata*and *A. tanaica* (0.003073). Monte Carlo (X^2) pairwise genetic comparison revealed highly significant differences between all species (P<0.001). In the Neighbour-joining tree, there were two main grouping, and in the first group, *A. caspia* and *A. f. nilotica* exhibited the closest genetic similarity which was the sister group to *A. immaculata*. *A .tanaica*seems to be the most divergent in this grouping. Another group contained only *A. maeotica* which showed the highest genetic differentiation among *Alosa* genus.

Keywords: Shad species, *Alosa* genus, mtDNA genes, systematics.

MtDNA Genleri Kullanarak Türk Deniz Sularında Tirsi Türlerinin (Alosa spp.) Moleküler Sistematik Analizi

Özet

Türk deniz sularından beş tirsi türünün (*Alosa caspia*, *A. fallax nilotica*, *Alosa maeotica*, *Alosa immaculata*, *Alosa tanaica*) filogenetik ilişkisi mitokondriyal DNA polimeraz zincir reaksyionu-restriksiyon parça uzunluk polimorfizmi ile araştırılmıştır. PCR ile uygulamaya tabi tutulmuş altı gen bölgesi; NADH 5/6, NADH 3/4 cytochrom b, COX, 16 S rRNA ve D-Loop, yedi restriksiyon enzimi ile (*Bsur*I, *Alu*I, *Ehe*I, *Hin6*I, *Rsa*I, *Xho*I *Bsh1236*I) sırasıyla kesilmiştir. Filogenetik analiz için bir araya getirilmiş altı genden toplam 45 haplotip beş tirsi türünden tespit edilmiş ve ortalama haplotip çeşitliliği ile türler içerisindeki nükleotit çeşitliliği sırasıyla 0,8809 ve 0,0022 olarak bulunmuştur. En yüksek genetik farklılık *A. caspia* ve *A. maeotica* (0,013727) arasında, en düşük *A. immaculata* ve *A. tanaica* (0,003073) arasında gözlenmiştir. Monte Carlo (X^2) ikili genetik karşılaştırma sonucunda tüm türler arasında yüksek derecede önemli farklılıklar ortaya çıkmıştır (P<0,001). Komşu katılımlı ağaçta oluşan iki ana grupta; birinci grup *A. caspia* ve *A. f. nilotica* genetik benzerlik olarak birbirine çok yakın görülürken, *A. immaculata*.bu gruba kardeş grup olmuştur. *A. tanaica* ise bu grubun içerisinde bir hayli farklı görünmektedir. *Alosa* cinsi arasında en yüksek genetik farklılaşma gösteren *A. maeotica* sadece diğer grup içerisinde yer almıştır.

Anahtar Kelimeler: Tirsi türleri, *Alosa* cinsi, mtDNA, sistematik.

Introduction

The Clupeidae is one of the world's most commercially important families of fish. The genus *Alosa* is classified in the family Clupeidae and comprised of seven genera and 31 species (Whitehead, 1985). All species of shad that are found in the Atlantic Ocean, and the Mediterranean,

Marmara, Black, Azov,and Caspian Seas are currently united under a single genus, *Alosa*. They are pelagic, predominantly anadromous fish and inhabit marine, estuarine and fresh water regions.

Generally only one species (*Alosa maeotica*) and six subspecies (*A. caspia bulgarica,Alosa caspia nordmanni, Alosa caspia palaeostomi, Alosa caspia tanaica, Alosa fallax nilotica, Alosa pontica pontica*) within the genus *Alosa*exist in the Black Sea on the basis of morphological characters (Slastenenko, 1956; Kuru, 1980; Geldiay andBalık, 1996; Demirsoy, 1998; Mater andBilecenoglu, 1999). However, Bilecenoglu *et al*. (2002) and Kuru (2004) revised the *Alosa* species and concluded that four species (*A. caspia, A. maeotica, A. tanaica, A. pontica*), and one subspecies (*A. fallax nilotica*) exist within the genus *Alosa* in the Black Sea on the basis of morphological characters. Moreover, Pontic shad *Alosa pontica* has been recently revised as *Alosa immaculata*by Kottelat (1997).

In the Black, Marmara, Azov and Caspian Seas, four *Alosa* species are endemic: Caspian shad *Alosa caspia* (Eichwald, 1838), Black Sea shad *Alosa maeotica* (Grimm, 1901), Pontic shad *Alosa immaculate* (Bennet, 1835), and Azov shad *Alosa tanaica* (Grimm, 1901). *A. caspia* is commonly found in the Black, Marmara, Azov and Caspian Seas. *A. maeotica* occurs in the Black Sea (western part) and Marmara Sea. *A. tanaica* is found in the southwest Black Sea. *A. pontica* is found along the south Black Sea coast and the Marmara and Azov Seas (Svetovidov, 1963; Aksiray, 1987; Whitehead, 1984). However, the subspecies twaite shad (*Alosa fallax nilotica* (Saint-Geoffrey, 1908) has a broad distribution expanding from the eastern Atlantic including the Mediterranean, the Marmara and Black Sea.

Despite the number of studies on the biology, distribution and the identification characteristics of the *Alosa* species (Svetovidov, 1964; Whitehead, 1985; Aksiray, 1987; Turan and Basusta, 2001; Erguden *et al*., 2007; Turan *et al*., 2007; Eschmeyer, 2014), little is known about their phylogenetic relationships resulting in a systematic and taxonomic uncertainty which may undermine the establishment of adequate conservation measures.

Rapidly evolving mitochondrial DNA genes have been shown to be a powerful tool for the assessment of phylogeographic patterns in anadromous and marine fishes (Meyer *et al*., 1990; Bernatchez and Wilson 1998; Turan *et al*., 2009a). The pattern of maternally inheritance and rapid rate of evolutionary change of mtDNA compared to nuclear DNA makes it a suitable tool to accomplish genetic studies of several fish groups at multiple taxonomic levels (Kocher and Stepien, 1997; Durand *et al*., 2002; Turan *et al*., 2009b).

In this study, six mtDNA gene segments (Cytochrome b, COX, D-Loop, ND3/4, ND 5/6 and 16S rDNA) were used to investigate the systematic relationships of four species (*A. caspia*, *A. maeotica*, *A. tanaica* and *A.immaculata*) and one subspecies (*A. fallax nilotica*) of the genus *Alosa*, and reveal the consistency of the mtDNA genes for the identification of *Alosa* species.

Materials and Methods

Shad specimens were collected using commercial gill net from the Mediterranean, and Black Seas. A total of 150 individuals from *Alosa caspia*, *Alosa fallax nilotica*, *Alosa maeotica*, *Alosa immaculata*, *Alosa tanaica*were collected. Taxonomic identification of the samples was carried out according to Whitehead (1985), Turan *et al*. (2007) and Eschmeyer (2014). The sampling details are given in Table 1. The samples were placed individually into plastic bags, and kept frozen at -20°C until transportation. Muscle tissue was taken from each individual in the field or in the laboratory and stored in 98% ethanol.

Total l DNA was extracted using a high-salt protocol (Sambrook *et al*., 1989).Mitochondrial DNA variation was analyzed by restriction fragment length polymorphisms (RFLPs) performed on PCR amplified products. Six mtDNA genes, Cytochrome b, COX, D-loop, ND 3-4, ND5-6, 16S rRNA, were amplified using universal primers. These mtDNA genes were chosen because they offer different levels of sensitivity for phylogenetic analysis. The list of universal primers is given in Table 2.

PCR amplification conditions were as follows: one preliminary denaturation 94°C for 5 min followed by 35 PCR cycles. Strand denaturation was made at 94 °C for 1 min, annealing at 52°C/20s 72°C, and primer extension at 72°C for 1.5 min. A final extension at 72°C for 5 min was performed. Same PCR conditions were used for all genes. The PCR cocktail used for all mtDNA geneswere;2.5 µl 10X polymerase buffer, 0.5 µl dNTP (10 mM), 0.3 µl Taq DNA polymerase (3 U/µl), 0.10 µl primers, 1µl template DNA, and water for a total reaction volume of 25 µl.

Amplified mtDNA segments from two individuals per population were digested with different restriction enzymes to check presence of recognition sites. The informative restriction enzymes were then applied to all species. Six restriction enzymes were used for each gene segment. The informative restriction enzymes, used in the analyses,were *Bsur*I *(Hae III)*, *Alu*I, *Ehe*I (*Nar*I),*Hin6*I(*Hha*I), *Rsa*I, and *Xho*I for the ND5/6 gene segment, and *Hae III*, *Hha*I,*Xho*I, *Alu*I, *Rsa*I, *Bsh*1236I (*FnuD*II)for the Cytb, COX, D-Loop,ND3/4 and 16SrDNA gene segments for all species. The fragments of the restricted DNA samples were separated on 6% polyacrylamide gels, together with a size marker. A silver nitrate staining protocol (Tegelström, 1987) was used to visualize the DNA fragments on gel.

Table 1. The samplingdetails of *Alosa* species

Species	Sampling Location	Geographic Coordinates	Collection Date	Gear	Sample size
Alosa caspia(AC)	Black Sea (Sile)	41º 10´ N 29º 39' E	19.11.2005	Gill net	30
A. fallax nilotica(AFN)	Mediterranean Sea (Mersin)	36º 48´ N 34º 41' E	04.12.2005	Gill net	30
Alosa maeotica(AM)	Black Sea (Sile)	41º 10' N 29º 37´ E	07.11.2005	Gill net	30
Alosa immaculata(AI)	Black Sea (Sile)	41º 10' N 29º 35' E	27.10.2005	Gill net	30
Alosa tanaica(AT)	Black Sea(Sile)	41º 10' N 29º 38´ E	18.11.2005	Gill net	30

Table 2. The structure of the universal primers used for mtDNA gene regions in the study

Gen Region	Primers
Cyt b	F: 5'-CCT TCT AAC ATT TCA GTC TGA TG-3'
	R: 5'-AGG ATT GTG GCC CCT GCA AAT AC-3'
COX	F: 5'-AGC CCA TGA CCT TTA ACA GG-3'
	R: 5'-GAC TAC ATC AAC AAA ATG TCA GTA TCA-3'
D-loop	F: 5'-CAC AGG TCT ATC ACC CTA TTA AC CA-3'
	R: 5'-CTG GTT CGT CCA AGT GCA-3'
ND3/4	F: 5'-TAA (C/T)TA GTA CAG (C/T)TG ACT TCC AA-3'
	R: 5'-TTT TGG TTC CTA AGA CCA A(C/T)G GAT-3';
ND5/6	F: 5'-AAC AGT TCA TCC GTT GGT CTT AGG-3'
	R: 5'-TAA CAA CGG TGG TTC TTC
16S	F: 5'-CG (CT) AAG GGA A (ACT) G CTG AAA-3'
	R: 5'-CCG GTC TGA ACT CAG ATC ACG TAG-3'.

The degree of nucleotide divergence was estimated using the REAP computer package (McElroy *et al*., 1992). Neighbour-Joining method (Saitou and Nei, 1987) was used for the construction of the phylogenetic trees. The confidence of the branches was evaluated by bootstrapping (1000 replicates) (Felsenstein, 1985). Trees were drawn using the MEGA5 program (Tamura *et al*., 2011). The degree of geographical heterogeneity of mtDNA haplotype distribution was assessed using X^2 statistics as described by Roff and Bentzen (1989). For restriction site data, Monte-Carlo randomization tests were performed to determine the significance of haplotype frequency distributions among sampling sites using MONTE program from REAP package(McElroy *et al*.,1992).

Results

The amplified segments of ND 5-6, ND3/4, Cyt b, 16S rRNA, Dloop, COX gene regions were approximately 2500, 2400, 2100, 2000, 1800, 1300 bp, respectively, which is corresponding to about 75% (12.1 kb in total) of a typical 16 kb mitochondrial genome (Wallace, 1986; Meyer, 1993).The restriction enzymes generated a total of 219 restriction sites, corresponding to an estimated average number of 948 bp surveyed (about 6% of total mtDNA). Heteroplasmy was not detected. Polymorphism was found in ND 5/6, Cyt b, D-loop and 16S rRNA genes. However, ND3/4 and COX genes were monomorphic for all species.

ND 5/6

The ND 5/6 mt DNA gene region generated 21 different haplotypes for five species.Seven restriction enzymes had at least one recognitionsite on the amplified ND 5/6 gene segments. The highest number of haplotypes (8 haplotypes) was observed for *A. f. nilotica* and *A.immaculata*, and lowest number of haplothpes (1 haplotypes) was observed for *A. caspia*. In the Neighbor Joining (NJ) tree, *A. caspia*and *A. maeotica*were clustered together and exhibited close genetic similarity to *A. immaculate*, *A. tanaica*was clustered together with *A. f. nilotica* that showed the highest genetic differentiation (Figure1).

Cyt b

The Cyt bgene region generated 18 different haplotypes for five species.Seven restriction enzymes had at least one recognition site on the amplified Cyt b gene segment. The highest number of haplotypes (4 haplotypes) was observed for *A.immaculate*, and the lowest (1 haplotypes) was observed for *A. maeotica*. In the NJ tree, *A. maeotica* seems to be the most genetically distinct species from the other four species. *A. caspia* and *A. maeotica* showed the highest genetic differentiation among *Alosa* genus (Figure 1).

D-loop

Six restriction enzymes generated 6 different haplotypes. In the NJ tree, *A. immaculate* seems to be the most genetically distinct species, while *A. caspia*

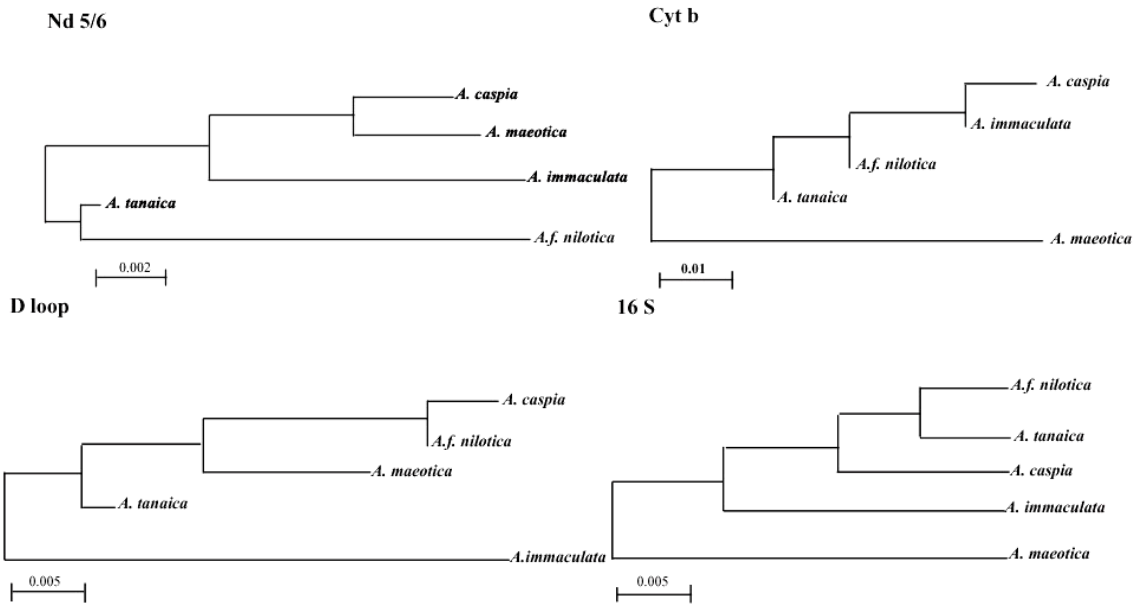

Figure 1. Neighbour-joining trees of the molecular systematic relationships amongthe five shad species of the genus *Alosa* for each gene.

and *A. f. nilotica* exhibited closest genetic similarity to *A.maeotica* which showed close phylogenetic relationship to *A. tanaica* (Figure 1).

16S rRNA

Six restriction enzymes generated 2 different haplotypes for five species. In the NJ tree, *A.f. nilotica* and *A. tanaica* exhibited closest genetic similarity and *A. caspia* showed close phylogenetic relationship to *A. f. nilotica* and *A.tanaica. A. maeotica* seems to be the most genetically distinct among the five species (Figure 1).

Combined mtDNA Genes

The combined polymorphic and monomorphic six mtDNA gene segments (ND 5/6, Cty b, 16S, D-loop, ND 3/4, COX) revealed45 different combined haplotypes for five shad species. The highest number of haplotypes (13 haplotypes) was observed for *A.f. nilotica*, and lowest number of haplotypes (4 haplotypes) was observed for *A. caspia* (Table 3). The different restriction patterns that can be used as diagnostic marker between the five species, and the total numbers of restriction patterns that can be used for species identification are presented in Table 3. The species-specific patterns, taken into consideration either individually or in various combinations, can be used to distinguish the five species under study. The average haplotype diversity and nucleotide diversity within the genus *Alosa* were 0.8809 and 0.0022 respectively. The average nucleotide diversity and nucleotide divergence among species were 0.009248 and 0.007080respectively. The highest genetic divergence was observed between *A. caspia* and *A. maeotica* (0.013727), and lowest between *A. immaculata* and *A. tanaica* (0.003073), and lowest nucleotide diversity (0.004902) was observed between *A. maeotica* and*A. Immaculate* (Table 4). In Monte Carlo pairwise comparisons highly significant differences (P<0.001) between all species were found (Table 4).In the NJ tree, *A. caspia* and *A. f. nilotica* exhibited closest genetic similarity which was the sister group to *A. immaculata*. *A. tanaica* was clustered after these species. The other line age contained *A. maeotica* which showed the highest genetic differentiation among the members of genus *Alosa* (Figure 2).

Discussion

In the present study, both separate and combined analyses of mtDNA genes resulted in different patterns of phylogenetic relationship among the species of *Alosa* genus. With combined analysis of all mtDNA genes, *A. caspia*, *A. f. nilotica*, *A. immaculata* and *A. tanaica* exhibited close genetic similarity and *A. maeotica* showed the highest genetic differentiation within *Alosa* genus. The nucleotide divergence of *A. maeotica*from the other *Alosa* species is about 0.01.This indicate that the congeneric divergence between the species of genus *Alosa* is low compared to other marine species such as herring (0.1; Shaw *et al*., 1999), mullets (0.2; Semina *et al*., 2007). The congeneric divergence was similar with horse mackerel (0.01; Turan *et al*.,2009a).Semina *et al*. (2007) reported that the pairwise sequence divergence estimated among the three mullet species based on RFLP analysis of 12S/16S rRNA data varied from 0.27 (between *M. cephalus* and *L. haematocheila*) to 0.10 (between *L. haematocheila* and *L. aurata*). *A. f.*

Table 3. Composite genotypes (haplotypes) and frequencies within the studied *Alosa* species

	Composite Haplotypes	AC	AFN	AM	AI	AT	Total
1.	AAAAAAAAAAAABCAAAAAAABAAAAAAAAAAAAAA	8					8
2.	AAAAAAAAAAAABAAAAAAAABAAAAAAAAAAAAAA	6					6
3.	AAAAAAAAAAAABCABAAAAABAAAAAAAAAAAAAA	9					9
4.	AAAAAAAAAAAABAAABAAAABAAAAAAAAAAAAAA	7					7
5.	AAAAAAAAAAAAACACAAAAAAAAAAAAAAAAAAAA		2				2
6.	BBABABAAAAAAACABAAAAAAAAAAAAAAAAAAAA		4				4
7.	BBBBBBAAAAAAAABAABAAAAAAAAAAAAAAAAAA		3				3
8.	AAAAAAAAAAAAAABBBBAAAAAAAAAAAAAAAAAA		2				2
9.	BBBBBBAAAAAAAABCABAAAAAAAAAAAAAAAAAA		2				2
10.	ABBBBAAAAAAAACABAAAAAAAAAAAAAAAAAAAA		2				2
11.	BAAAABAAAAAAAABCABAAAAAAAAAAAAAAAAAA		2				2
12.	BBABABAAAAAAAABAABAAAAAAAAAAAAAAAAAA		2				2
13.	BBABBBAAAAAAAABBBBAAAAAAAAAAAAAAAAAA		2				2
14.	BBABABAAAAAAAABCABAAAAAAAAAAAAAAAAAA		3				3
15.	AAAAAAAAAAAAAABAABAAAAAAAAAAAAAAAAAA		2				2
16.	BBBBBAAAAAAAACACAAAAAAAAAAAAAAAAAAAA		2				2
17.	BAAAAAAAAAAAAABAABAAAAAAAAAAAAAAAAAA		2				2
18.	AABABABBBBBBCBCCCBBBABAAAAAAAAAAAAAA			6			6
19.	AABAAABBBBBBCBCCCBBBABAAAAAAAAAAAAAA			5			5
20.	AAAABABBBBBBCBCCCBBBABAAAAAAAAAAAAAA			9			9
21.	AAAAAABBBBBBCBCCCBBBABAAAAAAAAAAAAAA			5			5
22.	BCACACBBBBBBCBCCCBBBABAAAAAAAAAAAAAA			2			2
23.	BCAABABBBBBBCBCCCBBBABAAAAAAAAAAAAAA			3			3
24.	BBAAAAAAAAABBAAAAAAAAAAAAAAAAAAAAAAA				2		2
25.	CCAAAAAAAAABAABAAAAAAAAAAAAAAAAAAAAA				6		6
26.	AAAAAAAAAAABBAAABBAAAAAAAAAAAAAAAAAA				4		4
27.	CCAAAAAAAAABBAAAAAAAAAAAAAAAAAAAAAAA				3		3
28.	ACAAAAAAAAABBAAAAAAAAAAAAAAAAAAAAAAA				3		3
29.	CAAAAAAAAAABBBAAABAAAAAAAAAAAAAAAAAA				3		3
30.	ACAAAAAAAAABBAAABBAAAAAAAAAAAAAAAAAA				3		3
31.	ACAAAAAAAAABBBAAABAAAAAAAAAAAAAAAAAA				2		2
32.	CCAAAAAAAAABBBAAABAAAAAAAAAAAAAAAAAA				2		2
33.	CAAAAAAAAAABBAAABBAAAAAAAAAAAAAAAAAA				2		2
34.	CAAAAAAAAAABABBBABAAACAAAAAAAAAAAAAA					2	2
35.	BAAAAAAAAAABABBBBBAAACAAAAAAAAAAAAAA					3	3
36.	CAAAAAAAAAABABBBBBAAACAAAAAAAAAAAAAA					3	3
37.	BABBBBAAAAABABABAAAAACAAAAAAAAAAAAAA					3	3
38.	CABAAAAAAAABABABAAAAACAAAAAAAAAAAAAA					4	4
39.	BABAAAAAAAABABBBABAAACAAAAAAAAAAAAAA					3	3
40.	BBBBBBAAAAABACBBABAAACAAAAAAAAAAAAAA						
41.	BBBBABAAAAABACBBABAAACAAAAAAAAAAAAAA					2	2
42.	CABAAAAAAAABABBBABAAACAAAAAAAAAAAAAA					2	2
43.	CAAAAAAAAAABACBBABAAACAAAAAAAAAAAAAA					2	2
44.	AAAAAAAAAAABACBBABAAACAAAAAAAAAAAAAA					2	2
45.	CAAAAAAAAAABABABAAAAACAAAAAAAAAAAAAA					2	2
	Total					2	2
		30	30	30	30	30	150
							AVR
	H	0.7701	0.9494	0.8276	0.9149	0.9425	0.8809
	S.E (+/-)	0.0247	0.0157	0.0329	0.0227	0.0160	0.0012
	N	0.0008	0.0033	0.0013	0.0023	0.0029	0.0022

Composite genotypes are denoted by capital letters in the following order. ND5/6: *Bsur*I *(Hae III*), *Alu*I, *Ehe*I (*Nar*I), *Hin*6I(*Hha*I), *Rsa*I, *and Xho*I; and Cyt b, 16S rRNA, D-loop, ND3/4,COX: *Hae III*, *Hha*I,*Xho*I, *Alu*I, *Rsa*I, *Bsh*1236I (*FnuD*II), H: Haplotip diversity, N:Nucleotid diversity, S.E.: Standard Error.

Table 4. Pairwise estimates of nucleotide divergence (below diagonal) and diversity (above diagonal) among the five *Alosa* species. *, significance value of Monte Carlo (X^2) test (P< 0.001)

Species	*A. caspia*	*A. f. nilotica*	*A. maeotica*	*A. tanaica*	*A. immaculata*
A. caspia	-	0.006640	0.005753	0.012301	0.006664
A. f. nilotica	0.003202 *	-	0.014901	0.006845	0.005281
A. maeotica	0.013727 *	0.012575 *	-	0.014389	0.004902
A. tanaica	0.003294 *	0.003987 *	0.012534 *	-	0.014803
A. immaculata	0.004764 *	0.003489 *	0.010155 *	0.003073 *	-

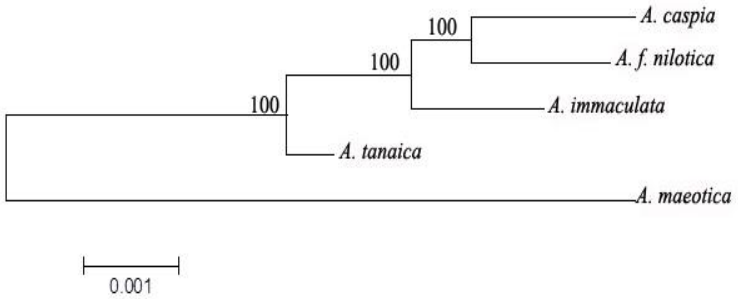

Figure 2. NJ tree based on nucleotide divergence. Numbers indicate the percentage out of 1000 bootstrap replicates that each node occurred in this majority-rule consensus tree.

nilotica and *A. caspia*clustered together in NJ tree seems to be the closest taxa in the genera. *A. immaculata*showed closergenetic relationship to *A. f. nilotica* and *A. caspia* than to *A. tanaica*.

In the present study, *A. f. nilotica* seems to be the putative sister species of *A. caspia*. This situation is also in agreement with the studies by Faria *et al*. (2006) based on the sequence analysis of two mtDNA (Cyt b, ND1) gene segments. Faria *et al*. (2006) mentioned the existence of ahighly divergent haplotype for *A. immaculata* and a closer relationship between *A. fallax* and *A. immaculata* were found. Bowen *et al*. (2008) similarly used mtDNA sequence analysis and also found the lowest genetic distance between *A. immaculata* and *A. f. fallax*.

The taxonomic status of the genus *Alosa* appears to be in conflict, the number of species in the genus remains in a state of flux. Considerable polymorphism in the genus has resulted in the recognition of numerous sub-species (Bagliniere *et al*., 2003). In this study, phylogenetic analysis of *A.caspia*, *A. fallax nilotica*, *A.maeotica*, *A.immaculata*, *A.tanaica* were carried out, and the pattern of relationship as well as the amount of genetic divergence between species were revealed. In light of current results, additional genetic markers such as single nucleotide polymorphism (SNP) markers that could be obtained easily from next generation sequencing should also be used to evaluate the phylogenetic relationship among Alosa species.

Alosa species are extremely vulnerable to anthropogenic changes, especially related to access and quality of their spawning grounds (Faria *et al*., 2006). Therefore, more conservation measures should be conducted to perpetuate the stocks of these species, especially the rarely found *Alosa* species such as *Alosa maeotica*, *Alosa immaculata*and*Alosa tanaica*.

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