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Allozyme Variation and Genetic Identification of Shad Species (Pisces: Clupeidae, Genus *Alosa*) Along Bulgarian Black Sea Coast

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Abstract: All Black Sea shads are listed in IUCN Red List as vulnerable species. The absence of correspondence between morphological and genetic features shows the complexity in genus *Alosa* taxonomy in Black Sea. To receive precise data for actual species status and respectively conservation of shads, the application of genetic methods are necessary.

A comparison between two shad species (*A. immaculata* and *A. caspia*) from Bulgarian Black Sea coast and Danube River was carried out on the basis of genetic-biochemical analyses. Twelve enzymes (encoded by 27 loci) and general muscle proteins and haemoglobin (encoded by 43 loci) were investigated. Polymorphic variation in some loci as *HB-1*, HB-2*, PROT-1**, *PROT-2*, PROT-3*, ADH**, *EST-3*, MDH-1*, MEP-1* PGM-2** and *SDH** can be used for analyses of *Alosa immaculata* populations. Lactate dehydrogenase (LDH) is a species specific marker for distinguishing *A. immaculata* and *A. caspia*. Genetic markers such as: 6-PGDH, SOD and PROT can be used to split the rare shad species (*A.* spp) found in Black Sea. Genetic distance (D_{Nei} = 0.038) between *A. immaculata* and *A. caspia* showed that they have quite recently divergated, while the distance between the two mentioned above species and *A.* spp was found to be higher (D_{Nei} = 0.066).

Key words: *Alosa*, Black Sea, genetic markers, electrophoresis, isoelectric focusing, enzymes, vulnerable species

Introduction

Clupeidae is one of the world's most commercially important families of fish. Despite their importance, little is known about the phylogenetic relationships within genus *Alosa*, resulting in systematic and taxonomic uncertainty, which may undermine the establishment of adequate conservation measures (Faria *et al*. 2006).

Kovachev (1922), Drensky (1923), Maiorova (1939), Pavlov (1959), Stoyanov *et al.* (1963), Marinov (1964) and Kolarov (1991 a, b and c) worked on the taxonomy of shad's species in Black Sea. Georgiev and Kolarov (1958) described *Alosa fallax nilotica*, Geoffroy as a new species in Bulgarian Black Sea coast. Banarescu (1964) split genus *Alosa* in two subgenera – *Alosa* and *Caspioalosa.* Based on morphological criteria, Svetovidov (1964) considered *Alosa* as a single genus with two subgenera (*Alosa* and *Pomolobus*) and rejected the validity of *Caspioalosa* as different subgenus.

Zivkov *et al.* (2005) and Vasil'eva (2007) reported that in Black Sea fish fauna the following species exist: *A. immaculata* (*A. pontica*), *A. caspia*, *A. meotica* and *A. fallax*.

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According to Vasil'eva (2007) *A. bulgarica* should be considered as a synonym of *A. caspia*.

Morphological identification of *Alosa* species in Black and Azov Seas often is impossible in view of the higher variability of morphological features (Banarescu 1964, Vasil'eva 2007, Mezhzherin *et al.* 2009) and additional specific analyses are needed.

The systematic work on the genus *Alosa* and clarification of the taxonomical position and species conservation status should rely on genetic analyses (Bianco 2002, Faria *et al*. 2006). Based on mt DNA analyses, Faria *et al.* (2006) provided a molecular phylogenetic overview of the genus *Alosa* showing that in some cases the species boundaries are not well defined and the application of multiple molecular markers are needed .

Allendorf, Seeb (2000) concluded that it is important to examine many loci when estimating genetic differentiation to infer historical amounts of gene flow and patterns of genetic exchange among populations. It is less important whether these loci are allozymes or nuclear DNA markers.

Taxonomic studies on genus *Alosa* covered allozyme, mitochondrial and nuclear DNA analyses to evaluate genetic differentiation between different species and understand the phylogenetic position of European and North American *Alosa* species (O'Maoileidigh *et al*. 1988, Boisneau *et al.* 1992, Bentzen *et al.* 1993, Alexandrino *et al.* 1996, Le Corre *et al.* 2005, Alexandrino *et al.* 2006, Faria *et al.* 2004 and 2006, Volk *et al.* 2007, Bowen *et al.* 2008, Mezhzherin *et al.* 2009, Coscia *et al.* 2010).

Mezhzherin *et al.* (2009) on the basis of allozyme data confirmed *Alosa immaculata*, *A. maeotica* and *A. caspia* conspecifity in Azov-Black Sea basin.

Species identification of Black Sea shads was problematic because of confusing morphological variation within some species.

Because of the fact that the shad species are vulnerable according to IUCN criteria, genetic analyses are needed in order to clarify the taxonomic position and the conservation status of the Black Sea shad species and their populations.

Specifically the goals were to determine genetic variability of *A. immaculata* populations in the Black Sea and to clarify species status of *Alosa immaculata* and *A. caspia* in Black Sea.

Material and Methods

During the period 1978-2010, 374 shad samples from Bulgarian Black Sea coast, Danube River (Fig. 1) and Rezovska River (one sample), as well as *Alosa caspia* from Caspian Sea (two samples) were collected and analyzed.

In the analyses of the enzymes and non-enzyme protein systems, a homogenates of white dorsal muscle, haemoglobin, eye (retina), liver and gonads were used.

Proteins were separated by horizontal starch gel electrophoresis according to Smithies (1955), modified by Dobrovolov (1973).

Isoelectric focusing (IEF) on thin polyacrylamide Ampholone gel with pH gradients between 3.5-10.0 and ultrathin polyacrylamide Servalyte gel plates with the equipment of LKB (Stockholm, Sweden) was used. The proteins were stained with Commassie Brilliant Blue R-250.

The following twelve enzymatic systems were studied: alcohol gehydrogenase (EC 1.1.1.1. – ADH), esterase (EC 3.1.1.1 – EST), lactate dehydrogenase (EC 1.1.1.27 – LDH), malate dehydrogenase (EC 1.1.1.37 – MDH), malic enzyme (EC 1.1.1.40 – MEP), superoxide dismutase (EC 1.15.1.1 – SOD), glucose-6-phosphate dehydrogenase (EC 1.1.1.49 – G6PDH), phosphoglucomutase (EC 5.4.2.2. – PGM), alkaline phosphatase (EC 3.1.3.1 – ALP), isocitrate dehydrogenase (EC 1.1.1.42 – IDHP), sorbitol dehydrogenase (EC 1.1.1.14-SDH), phosphogluconate dehydrogenase (1.1.1.44 – PGDH). Staining of different enzymes was performed according to Shaw and Prasad (1970). The buffer systems of Dobrovolov (1976) and Clayton and Gee (1969) were used for the electrophoresis. The nomenclature of

loci and alleles used here followed essentially the recommendation of Shaklee *et al.* (1990). Genetic distance (D_{Nei}) was calculated according to Nei (1972).

Results

General muscle proteins (PROT)

Starch gel electrophoresis detected 19 protein fractions but species differentiation and polymorphism in the species compared was not found (Fig. 2).

Black Sea shads (*A. immaculata, A. caspia* and *A. maeotica*), analyzed in the present study, were identified morphologically by one of the authors (Zh. Georgiev), but their electrophoretical spectra did not show species specific differences, which confirmed morphological variety.

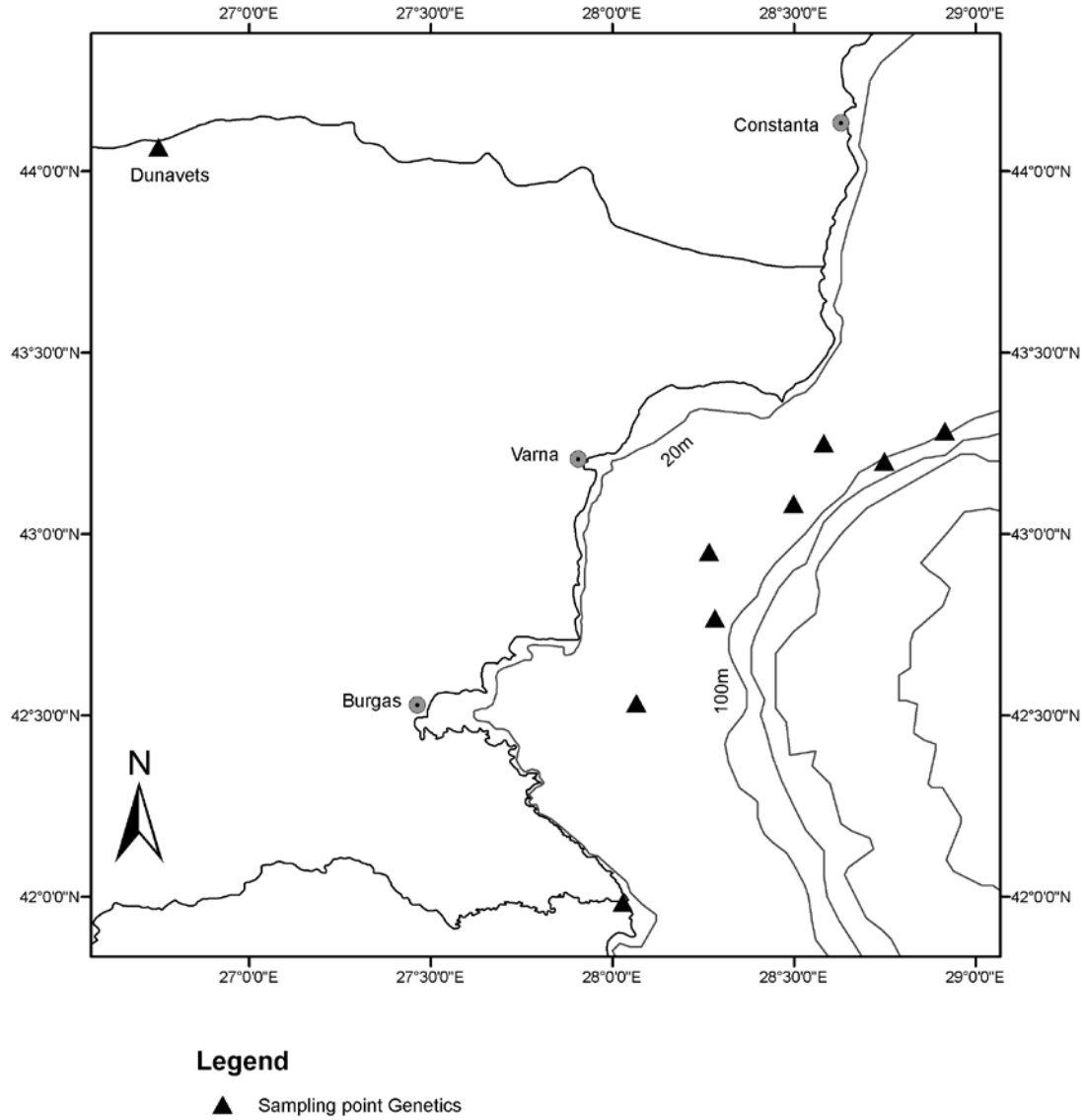

Fig. 1. Sampling localities of *Alosa immaculata*.

The isoelectric focusing spectra on thin and ultrathin polyacrilamide Ampholine and Servalite gel plates showed 40-41 general muscle protein fractions. Differences between the electrophoretical spectra of *A. immaculata*, *A. caspia* and *A.maeotica* were not found, with the exception of several specimens (Fig.3, N9), which belong to the *A.* spp. species.

Using isoelectric focusing (IEF) on muscle tissue, polymorphic variations in three loci (*PROT-1*, PROT-2** and *PROT-3**) were detected. Polymorphism in *A. immaculata* was observed in Varna Bay, Kaliakra and in front of Kamtchiya River (Table 1). The criteria χ^2 did not increase 3.84, which is evidence for genetic equilibrium in the populations analyzed. Only in one sample (Varna Bay) on *PROT-2** a higher value of χ^2 criteria (χ^2=4.883, df=1) was registered. IEF spectra of general muscle proteins in the eye tissue (retina) did not show differences between the species compared.

Haemoglobin (HB). Black Sea shads showed 16-17 intensively coloured haemoglobin fractions using isoelectric focusing (Fig. 4). Two polymorphic zones (*HB-1** and *HB-2**) were observed in the samples, caught in front of Kamtchiya River, Cape Kaliakra and Nesebar (Table 1). The *HB-1** polymorphism showed significant differences in gene frequencies in the samples caught in front of Kamtchiya River and Nesebar. Differences between the species compared were not found (Fig. 4).

Lactate dehydrogenase (LDH). Twelve fractions on starch gel on LDH enzyme system were observed in the Black Sea shads. The two duplicated

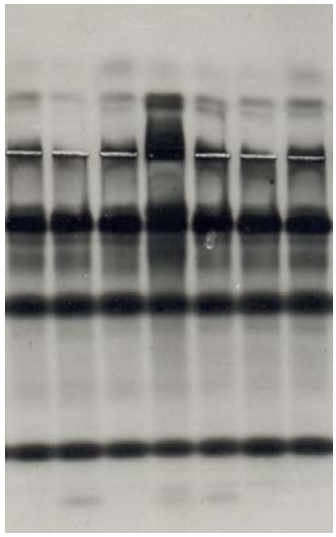

Fig. 2. Starch gel electrophoresis (general muscle proteins PROT): 1-3 *Alosa immaculata* and 4-7 – *A. caspia*, 0 – origin.

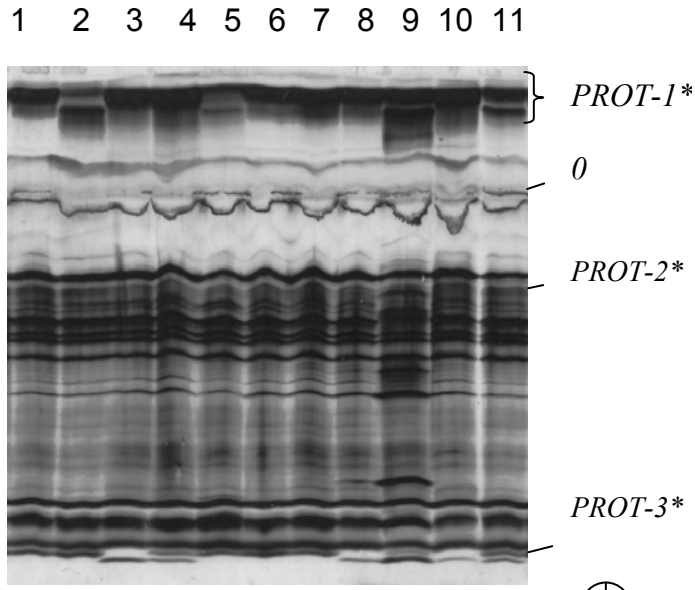

Fig. 3. Isoelectric focusing (IEF) on thin polyacrilamide Ampholine gel plate on general muscle proteins, with pH range 3-10: 1 – 8 and 10-11 – *Alosa immaculata* and 9 – *A.* spp., 0 – origin.

Table 1. Calculated allele frequencies of established polymorphic loci in general muscle protein, haemoglobin, alcochol dehydrogenase, esterases, malate dehydrogenase and malic enzyme, phosphoglucomutase and sorbitoldehydrogenase on the *A. immaculata*, from different localities: a*- is slower (more cathodal), and b*- faster (more anodal) electromorph, n – number of individuals analyzed.

Locus	**Allele**		**Allele**	**frequencies**		
		Varna Bay N=52	Cape Kaliakra n=92	Danube River n=57	In front of Kamchiya River, n=46	Nessebar n=42
*PROT-1**	a* b*		0.364 0.636			
*PROT-2**	a* b*	0.595 0.405				
*PROT-3**	a* b*		0.273 0.727			
*HB-1**	a* b*				0.809 0.191	0.500 0.500
*HB-2**	a* b*		0.220 0.780			
ADH*	a* b*		0.891 0.109			
*EST-3**	a* b * c* d*		0.043 0.913 0.022 0.022			
*sMDH-1**	a* b* c*	0.397 0.588 0.015	0.380 0.598 0.022			
*sMEP-1**	a* b*		0.114 0.886	0.070 0.930		
*PGM-2**	a* b*					0.972 0.028
*SDH-1**	a* b*	0.978 0.022				

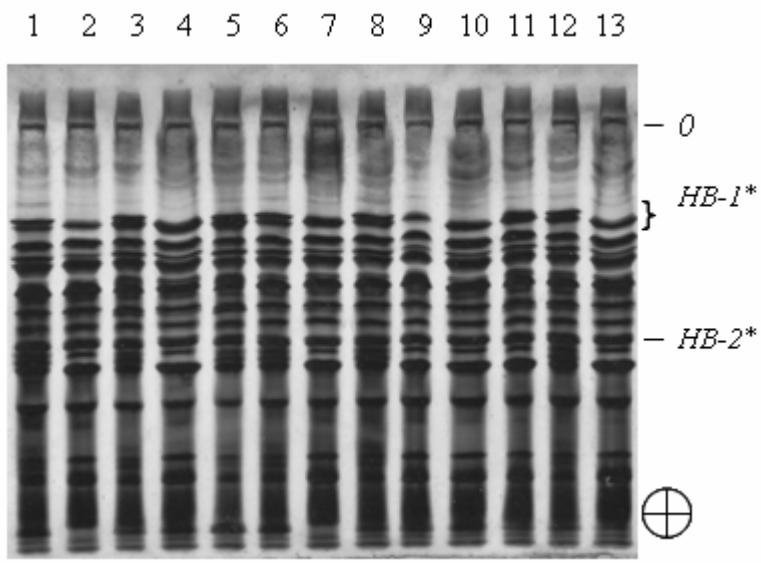

Fig. 4. Isoelectric focusing (IEF) on thin polyacrilamide Ampholine gel plate on haemoglobin (HB) with pH range 3-10: 1-6 – *Alosa immaculata* and 7-13 – *A. caspia* , 0 – origin.

genes *LDH-A*1* and *LDH-A*2* were established (Fig. 5A, B). Differences in the electrophoretic mobility of *LDH-A*1* and *LDH-A*2* between *A. immaculata* and *A. caspia* were registered (Fig. 5); they reflected on their hybrid interloci fractions. Because of the fact that hybrid fractions between the faster and slower electromorph were not found, we supposed that they belong to two nominant species.

The comparison between Black Sea species and two *A. caspia* specimens, caught in Caspian Sea (Fig. 5A) was made to determine which LDH spectrum belongs to which species. The faster *LDH-A*1* and *LDH-A*2* electromorphs were typical for *A. caspia*, while the slower – for *A. immaculata.* Both species were easily distinguished using the same enzyme system, analysed by isoelectric focusing (IEF), (Fig.5B).

The differences in electrophoretical mobility of all enzymes and non-enzymatic protein systems between *A. maeotica* and the mentioned above species were not registered. For this reason we compared only *A. immaculata* and *A.caspia*.

After analyses of eye tissue (retina) of Black Sea shads a new duplicated *LDH*C* locus (*LDH*C1* and *LDH*C2*) was found (Fig. 6). No differences in the electrophoretic mobility of *LDH-C** loci in the two species (*A. immaculata* and *A. caspia*) were found.

Malate dehydrogenase. Two loci with malate dehydrogenase activity (*sMDH-1** and *sMDH-2**) were observed. Polymorphism was found only in one of them (*sMDH-1**) (Fig. 7) in the *A. immaculata* samples from Cape Kaliakra and Varna Bay with close values of genetic frequencies (Table 1).

Malic enzyme. Two fractions, determined from two loci (*sMEP-1** and *sMEP-2**) were registered. Polymorphism was present only in one locus (s*MEP-1**) in the samples from Cape Kaliakra and Danube River (Table 1).

Esterases. Non-specific esterases were polymorphic in the shad species. In the **muscle** tissue six zones with esterase polymorphism, most likely determined from six loci were observed. Differences in the electrophoretical spectra of *A. immaculata* and *A. caspia* were not found. In the third zone (*EST-3**) polymorphism with four alleles was found (Table 1). In the **liver** esterase four zones were presented. In the first and fifth loci two allele polymorphism with null allele was found. Second and fourth zones were monomorphic, while the third zone was polymorphic with fourth allelic systems of inheriting. **Eritrocite** esterases showed four fractions. Allozyme differences between the two morphologically determined shad species were not found.

Phosphoglucomutase. Two zones, probably controlled from two genes (*PGM-1**and *PGM-2**) were observed. Polymorphism was found in the second locus (Table 1) only in Nesebar samples. Differences in this enzyme system between the species were not found.

6-Posphogluconat dehydrogenase (6-PGDH). Five zones, determined from five loci were estab-

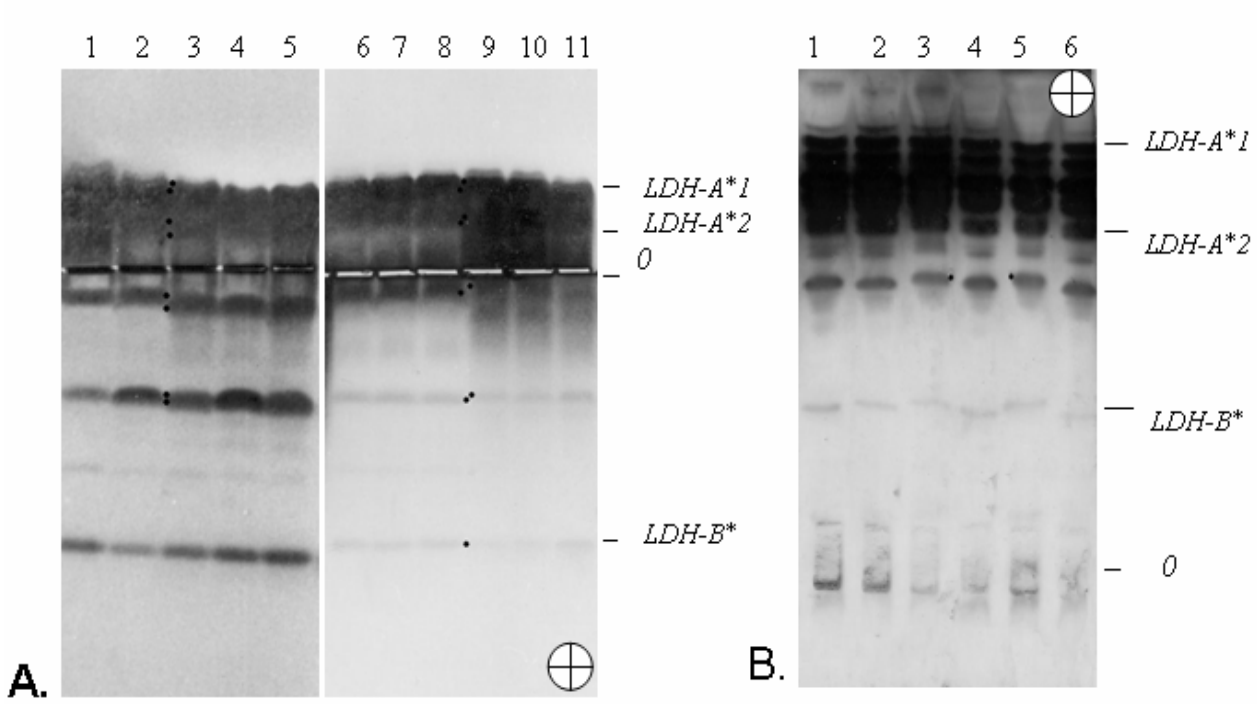

Fig. 5. A. Zymograms on Lactate dehydrogenase on starch gel: 1-2 – *A. immaculata*, 3-5 – *A. caspia* (Black Sea) , 6-8 – *A. caspia*, (Caspian Sea), 9-11 – *A. immaculata* (Black Sea); **B.** Isoelectric focusing (IEF) of LDH on thin polyacrilamide Ampholine gel plate with pH range 3-10: 3 and 5 – *Alosa immaculata*, 1, 2,4 and 6 – *Alosa caspia* , 0 – origin.

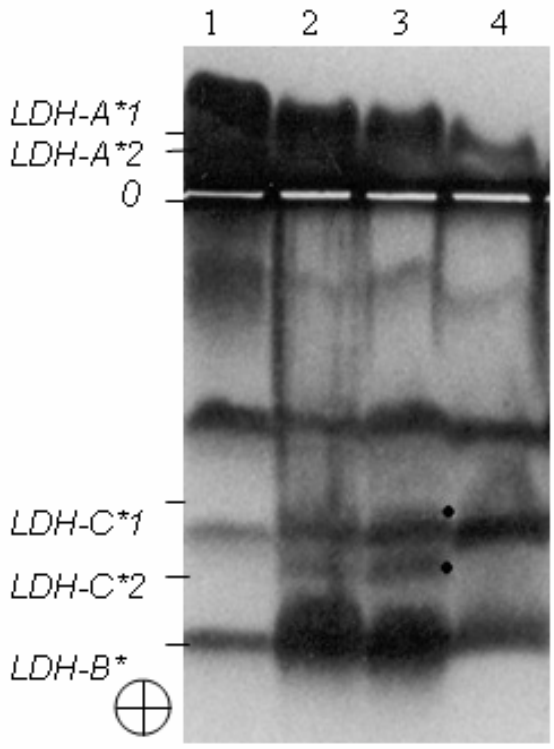

Fig. 6. Zymograms of LDH on starch gel by *A. immaculata*, Danube River, 1 – liver tissue, 2-3 – eye tissue, 4 – gonads tissue. The two new markers (*LDH-C*1* and *LDH-C*2*) were pointed with black spots, 0 – origin.

lished. Only two specimens, determined morphologically as Caspian shad (one with spots and one without spots), showed species specific differences in these enzyme systems which split them clearly from *A. immaculata* and *A. caspia*. (Fig. 8). We supposed that this other species (*A.* spp.), different electrophoretically from *A. immaculata* and *A. caspia* could probably be *A. fallax*. The species specific differences were also observed in the superoxide dismutase (Fig. 9).

Polymorphic systems such as **alcohol dehydrogenase** (**ADH**) and **sorbitoldehydrogenase** (**SDH**) in the liver tissue (Table 1) were established. Three monomorphic enzyme systems such as **superoxiddismutase (SOD)** – one zone (Fig. 9), **alkaline phosphatase (APL) –** three zones and **glucose-6-phosphate dehydrogenase (G-6PDH) –** two zones on starch gel were observed. Electrophoretical differences in the mentioned above enzyme systems between *A. immaculata* and *A.caspia* were not found.

The sample from Resovo River did not show electrophoretical differences from *A. caspia*. This supports Vasi'eva's (2007) opinion that *A. bulgarica* should be included in *A. caspia* synonyms.

The expected heterozygosity of different *A. immaculata* populations (according to Table 1) ranged

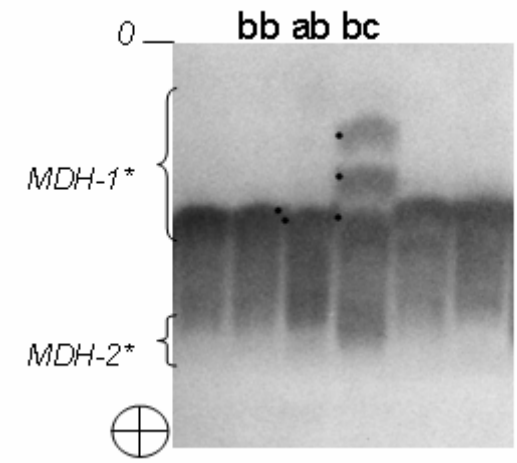

Fig. 7. Zymograms on malate dehydrogenase on starch gel by *A. immaculata*. Polymorphism in *MDH-1** locus – bb, ab and bc – phenotypes, 0 – origin.

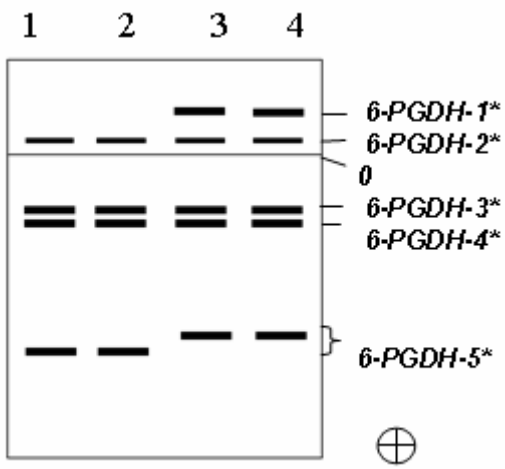

Fig. 8. Scheme on 6-phosphogluconate dehydrogenase on starch gel: 1 – *A. immaculata*, 2 – *A. caspia*, 3 – *Alosa* spp.(with spots) and 4 – *A. spp*. (without spots).

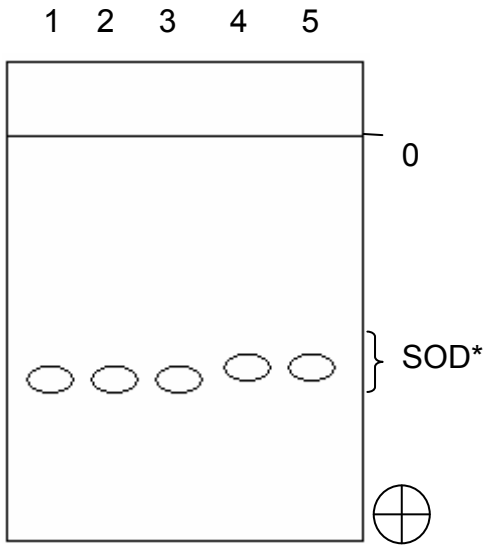

Fig. 9. Scheme on superoxide dismutase (SOD) on starch gel: 1-2 *A. immaculata*, 3 – *A. caspia*, 4-5 – *Alosa* spp., 0 – origin.

from 0.044 to 0.5. Percentage of polymorphic loci for general muscle proteins (PROT) and hemoglobin (HB) was 11.6, while for enzyme systems analyzed – 22.2 respectively. The average number of alleles per locus was calculated – 0.357. Genetic distance (D_{Nei}) between *A. immaculata* and *A.caspia* was 0.038, while the distance between the two mentioned species and *A.* spp. was 0.066.

Discussion

The taxonomic status of genus *Alosa* now appears stable but the number of species in the genus remains in a state of flux (Bowen *et al.* 2008). Morphological features are not indicative for species identification because of their high variability (Banarescu 1964; Mezhzherin *et al.* 2009; Vasil'eva, 2007). Therefore the application of genetic analyses for taxonomical identification of shad species in the Black Sea was needed. Mezhzherin *et al.* (2009) using electrophoresis in a polyacrylamide gel found polymorphism only in seven loci, which had rare alternative allele variants, and some of the loci found to be invariant in the Black Sea species. Our data presented in this study covered 12 polymorphic loci on the basis of isoelectric focusing (IEF) and starch gel electrophoresis. The polymorphism observed can be used for analyses of *A. immaculata* population structure in Black Sea.

In general, the samples determined morphologically as *A. immaculata* and *A.caspia* have no differences in their electrophoretical specters with the exception of LDH spectra. Hybridization between the two species was not found.

During the long period of electrophoretical investigations (1965-2010) on more than 207 marine and freshwater fish species we found that only in shad species (*A. immaculata* and *A. caspia*) in the Black Sea morphological differences did not correspond with the genetic ones, despite the large number of loci analyzed.

Based on the differences in the elctrophoretical LDH specters, Smith and Robertson (1981) proved the existence of new sprat species in New Zealand, which was mixed mechanically and morphologically indiscernible from the typical sprat species – *Sprattus antipodum*. This suggested that the lactate dehydrogenase enzyme system could be used as a genetic marker for species identification.

The existence of rare presented shad species (*Alosa* spp., Fig. 8 and 9) in the Black Sea based on

the differences in 6-PGDH, SOD and PROT spectra was proven. We assumed that *A.* spp. was probably *A. fallax*.

The polymorphism calculated in all loci analyzed, showed genetic equilibrium in the shad's population, which was probably the result from very close gene frequencies in the two species. Only in one case of *PROT-2** divergence from the gene equilibrium was registered, which was an indicator for the existence of two mixed populations. Because of close values of genetic frequencies in *sMDH-1** loci (Table 1) in Varna and Kaliakra populations, we supposed that probably the population analyzed was one and the same.

The investigated enzymes and non enzyme protein systems in the two species compared (*A. immaculata* and *A. caspia*) showed no differences, and the low value of genetic distance (D_{Nei} =0.038) proved that *A. caspia* and *A. immaculata* divergated not too long ago. The genetic distance between the two species and *A.* spp. (D_{Nei} =0.066) was higher. Based on the genetic distance and time of divergence calculated between *A.* spp. and *A. immaculata* and *A. caspia,* we supposed that this rare species was probably *A. fallax*. The relatively recent separation of Black Sea shad was also proven with molecular evidence (Faria *et al*. 2006).

Faria *et al*. (2006) proved a significant nucleotide divergence between *A. alosa* and *A. fallax,* but it was impossible to draw a distinct genetic boundary between the latter and *A. immaculata*.

Mezhzherin *et al*. (2009) on the basis of biochemical-genetic analyses proved the high degree of monomorphism between Azov-Black Sea shads (*A. immaculata*, *A. caspia* and *A. maeotica*) and confirmed their conspecifity. According to Faria *et al*. (2006) the relationship between the Black Sea species may be much more complex, as a variety of forms which was described by Mezhzherin, (pers. comm.), and later published in Mezhzherin *et al.* (2009).

According to the species specific genetic marker (LDH) established for the Black Sea shad species (*A. immaculata* and *A. caspia*) we assumed that they could not be included in a conspecyfity of one single species.

Further study of genus *Alosa* in the Black Sea should include analyses on additional markers (allozyme and DNA) and morphological features to clarify species status and phylogeny of the genus.

Conclusions

The polymorphism observed in some loci could be used for analyses of *A. immaculata* population structure in the Black Sea.

A genetic marker (LDH) for distinguishing of *A. immaculata* and *A. caspia* in Black Sea was established. New duplicated loci *LDH-A** and *LDH-C** were found.

The genetic distance between *A. immaculata* and *A. caspia* showed that they have quite recently divergated.

A rare species *A.* spp. (probably *A. fallax*) in the Black Sea was proven to be present with the application of genetic markers (6-PGDH, SOD and PROT).

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