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Protein Biomarkers for Identification of Some Gobiid Species (Actinopterygii: Gobiidae) along the Bulgarian Black Sea Coast

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Abstract: A comparison between twelve gobiid species from the Black Sea coast, coastal lakes and rivers was carried out on the basis of genetic-biochemical analysis (starch gel electrophoresis and isoelectric focusing on thin and ultrathin gel plates). Eleven enzymes and one non-enzyme protein system on starch gel (encoded by 29 loci) and general muscle protein loci on IEF (encoded by 22 loci) were studied. A single registered case of interspecies hybridization between *Neogobius* (*Apollonia*) *fluviatilis* and *Neogobius syrman* in Mandra Lake is proof that hybridization among this group happens only accidentally because of the fact that gobies exhibit parental care toward the eggs. First data for genetic-biochemical characteristics of *Knipowitschia longecaudata* are reported. Genetic distances (D_{Nei}=1.10) between *Knipowitschia caucasica* and *K. longecaudata* showed species specific differences. Genetical markers found for both species can be used for fast and precise identification. The received data are useful for the assessment of population status, taking into consideration that both species are threatened with extinction.Polymorphism on *EST-3*, EST-4*, LDH-B*, sMEP-1*, sMEP-2** and *MDH-1** loci can be used for the assessment of population infrastructure of *Neogobius* (*Apollonia*) *melanostomus*, *Gobius niger*, *Pomatoschistus marmoratus* and *K. caucasica*. The mobility of electrophoretical patterns of *LDH*B* locus were used as markers in determining the genus status of *Gobius ophiocephalus*. The hypothesis that *N. fluviatilis* and *N. melanostomus* belong to the subgenus *Apollonia* was confirmed using *LDH-B** locus as a genetic-biochemical marker. The four new enzymic systems (*ADH*, *GPI, GLUDH* and *G3PDH*) applied in the gobiid taxonomy gave species-specific spectra and can be used successfully as protein biomarkers. Their significance for the precise identification of species and populations, especially of those with minor morphological differences, as well as for the study of invasion pathways can increase in the future. Taxonomic investigations based on genetic analyses will contribute to biodiversity protection and conservation, to restoration and conservation of vulnerable gobiid populations and to sustainable fishery.

Key words: protein biomarkers, enzymes, IEF, electrophoresis, Gobiidae, Black Sea

Introduction

In the Bulgarian fish fauna, the family Gobiidae is represented by a comparatively large number of species. The main reference was published by Georgiev (1966), who described 23 species which belong to the genera *Aphia* Risso, 1826, *Pomatoschistus* Gill, 1864, *Knipowitschia* Iljin, 1929, *Chromogobius* Steindachner, 1863, *Gobius* Linnaeus, 1758, *Neogobius* Iljin, 1927, *Mesogobius* Bleeker, 1874, *Proterorhinus* Smitt, 1899, *Benthophiloides* Beling et Iljin, 1927 and *Benthophilus* Eichwald, 1831.

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Recently, 24 species were reported (Vassilev *et al.* 2011, 2012). Some species described earlier do not occur Bulgarian waters, or their records are probably due to misidentifications. The Ponto-Caspian endemics *K. longecaudata*, which had been reported as extinct for the Bulgarian coastal area (Stefanov 2006), was recently confirmed to be found in the lakes Shabla-Ezerets and Durankulak (Northern Black Sea coast) (Vassilev *et al.* 2012). Besides, *K. caucasica* showed high intraspecific variability of some important morphological characters (especially lateral line canals and coloration) (Georgiev 1964, 1966, Economidis, Miller 1990, Ahnelt *et al.* 1995, Kovačić, Pallaoro 2003).

Distinguishing taxonomic traits within the family Gobiidae include various kinds of characters: morphological, physiological, molecular, behavioral, ecological and geographic. Morphological and molecular characters are almost the only commonly used ones in the contemporary study of gobies (Kovačić, Pallaoro 2003). Using molecular systematics of gobioids will allow us to better tackle comparative evolutionary questions ranging from biogeography, evolution and diversification patterns of these fishes. Because of their small size and of the paucity of taxonomically informative morphological characteristics, the gobies are still viewed as one of the most difficult fish groups to classify and identify (Winterbottom 1984, in Sorice, Caputo 1999). In this context the electrophoretic analysis of proteins has rapidly gained acceptance as a systematic aid in conjunction with the usual morphological studies and has been particularly successful in elucidating taxonomic and phylogenetic relations (Sorice, Caputo 1999). Rüber, Agorreta (2011) described the most commonly used molecular markers in systematic studies of gobioids as follows: allozymes/isozymes (38 studies), mitochondrial cob gene (32 studies) and microsatellites (23 studies).

Genetic analysis based on allozyme data is a highly established quick and cheap method for species identification, which can be applied even on a piece of fillet sampled from the market (Martinsohn 2011). 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 those loci are allozymes or nuclear DNA markers. Genetic divergence and adaptation of local populations represent a potential resource for breeding programs in aquaculture and fishery management (Philips, Rab 2001). Iljin (1930), Krjizhanovski, Pchelina (1941), Pinchuk (1976, 1977) and Vasiljeva (1989) investigated the classification of Gobiid genera in the Black Sea basin. Shulman, Kulikova (1966), Kulikova, Prohodko (1970), Tesio, Mester (1970) examined electrophoretically some gobiid species but only on the basis of analyses of serum albumins and sarcoplasmic proteins. Iljin (1930), Banarescu (1964), Simonovič (1999) and Vasiljeva (2007) assumed that *N. melanostomus* had a subgeneric status as *Gobius* (*Apollonia*) *melanostomus* or *Neogobius* (*Apollonia*) *melanostomus*.

Protein and molecular features of different gobiid species from the Mediterranean, Atlantic Ocean, North Sea and the Black Sea were also investigated previously (Wallis, Beardmore 1984a,b, Dobrovolov *et al*., 1995, Dobrovolov 1996, Dougherty *et al*., 1996, Sorice, Caputo, 1999, Gysels *et al*., 2004a,b, Stefanni *et al.* 2003, Pampoulie *et al*. 2004). Dobrovolov *et al*. (1995) analysed electrophoretically myogens, hemoglobin and seven enzymic systems of 19 representatives of the family Gobiidae and proved that the electrophoretical spectra of myogens, hemoglobins, esterases, malate dehydrogenase, superoxiddismutase and phosphogluconat dehydrogenase are species-specific. On the basis of genetic analyses, the same authors specified that the species belonging to *Gobius*, *Neogobius*, *Mesogobius* and *Zosterisessor* should belong to the three genera: *Gobius*, *Neogobius* (subgenera: *Mesogobius* and *Ponticola*) and *Apollonia*. Dobrovolov, Rudnitcka (1994) observed lactate dehydrogenase polymorphism in *G. niger* along the Bulgarian Black Sea coast and the coast of the Northern Caucasus. The obtained polymorphism was recommended as a genetic marker for the investigation of population structure in the whole distribution area of this species.

Mitochondrial DNA cytochrome b gene analyses were applied for phylogeography and population genetic studies of round goby, *Apollonia melanostoma*, and tubenose goby, *Proterorhinus marmoratus*, from the Ponto-Caspian region of Eurasia to the North American Great Lakes (Dillon, Stepien 2001, Stepien, Tumeo 2006, Bowen, Stepien 2008, Sorokin *et al.* 2011).

Recently a significant expansion of the distribution of some Black Sea gobiid species was reported,

especially in the Lower and Middle Danube (Harka, Bíró 2007, Polačik *et al.* 2008), even to the Aral and Baltic Seas and to the North American Great Lakes (Stepien, Tumeo 2006). This invasive colonisations increased the ecological and economic importance of these gobies and imposed the need for new data concerning their genetics, ecology and invasiveness.

Seventeen species of the Bulgarian gobiid fauna are of conservation significance according to various national and international documents. Seven species are enlisted in the new Bulgarian Red Book (2011), 11 – in the Black Sea Red Book (Dumond, 1999), and 8 are included in the IUCN Red list (Vassilev *et al.* 2011). Simultaneously, gobies are of commercial significance to the countries bordering the Black and Azov Seas.

The aim of the present study is to establish new genetic-biochemical markers for gobiid species identification along the Bulgarian Black Sea coast, the Danube River, inland rivers and coastal lakes in connection with the species conservation status and potential invasiveness.

Material and Methods

During the period 2009–2010, the samples from the Bulgarian Black Sea coast, coastal lakes and rivers were collected and examined (Fig. 1, Table 1).

The samples of *Neogobius syrman* from the Dniester River and *Neogobius platyrostris* from the Black Sea, Odessa, analysed earlier, were used for comparison. Species identity was determined according to Miller (2003).

For the analysis of the enzymes and non-enzyme protein systems, a homogenate of white dorsal muscle and heart was used. Proteins were separated by horizontal starch gel electrophoresis according to Smithies (1955) methods, modified by Dobrovolov (1973). Isoelectric focusing (IEF) on thin polyacrilamide Ampholine gel with pH gradients between 3.5-10.0 was applied, as well as IEF on ultrathin polyacrylamide Servalyte gel plates provided by LKB (Stockholm, Sweden). The proteins were stained with Commassie Brilliant Blue R-250. Staining of different enzymes was performed according to Shaw, Prasad (1970). Buffer systems of Dobrovolov (1976) and Clayton, Gee (1969) were used for the electrophoresis. The nomenclature of mentioned loci and alleles followed the recommendations of Shaklee *et al.* (1990). Calculation of indices of genetic similarity and genetic distance was performed after Nei (1972). The loci were named from the most cathodal to the most anodal position.

The following five 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), glutamate dehydrogenase (EC 1.4.1.- GLUDH), glucose-6-phosphate dehydrogenase (EC 1.1.1.49 – G6PDH), glucose-6-phosphate isomerase (EC 5.3.1.9 – GPI), phosphoglucomutase (EC 5.4.2.2. PGM) and Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8 – G3PDH).

Results and Discussion

The interspecific comparison of the general muscle protein fractions (PROT) on the starch gel plates (Fig. 2A), as well as isoelectric focusing on thin and ultrathin gel plates (IEF) (Fig. 2B and 3) of the examined gobiid species demonstrated significant differences in the mobility of their electrophoretical patterns. The samples of one and the same species collected at different localities had similar electrophoretic patterns (Fig. 3).

The general muscle protein patterns of one individual from Mandra Lake showed hybridization between *N.* (*Apollonia*) *fluviatrilis* and *N. syrman* (Fig. 4). This is the first hybrid we registered throughout a long genetic observation of gobiid species. Although gobiid hybrids were detected before (Mukai *et al.*, 2000, Huyse *et al.*. 2004), this is the first time where a natural hybrid has been registered between different genera in this particular family.

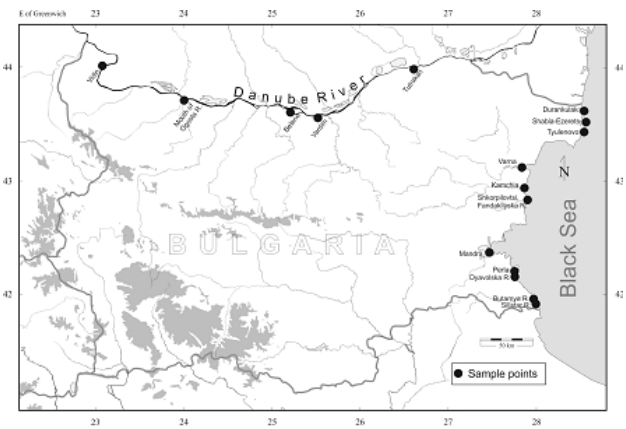

Fig.1. Sampling localities of gobiid species in Bulgaria

Table 1. Location and number of samples analysed for protein identification of some gobiid species

Species	Locality (number)				
Knipowitschia caucasica	Fandaklijska River (20)				
Knipowitschia longecaudata	Shabla-Ezerets Lake (20)				
Pomatoschistus marmoratus	Perla Bay (30)				
Gobius niger	Shkorpilovtsi (35)	Tyulenovo (35)	Varna Lake (39)		
Gobius ophiocephalus	Atiya Bay (10)				
Proterorhinus marmoratus	Shabla Lake (8)				
Neogobius (*Apollonia*) *melanostomus*	Shkorpilovtsi (10)	Shabla Lake (10)	Durankulak Lake (10)	Varna Bay (10)	Danube River (10)
Neogobius (*Apollonia*) *fluviatilis*	Varna Bay (7)	Danube River (7)	Silistar River (7)	Mandra Lake (7)	Butamya River (7)
Neogobius gymnotrachelus	Kamchiya River (10)	Djavolska River (10)			
Neogobius batrachocephalus	Varna (10)				
Neogobius syrman	Dniester River (5)				
Neogobius platyrostris	Black Sea, Odessa (12)				

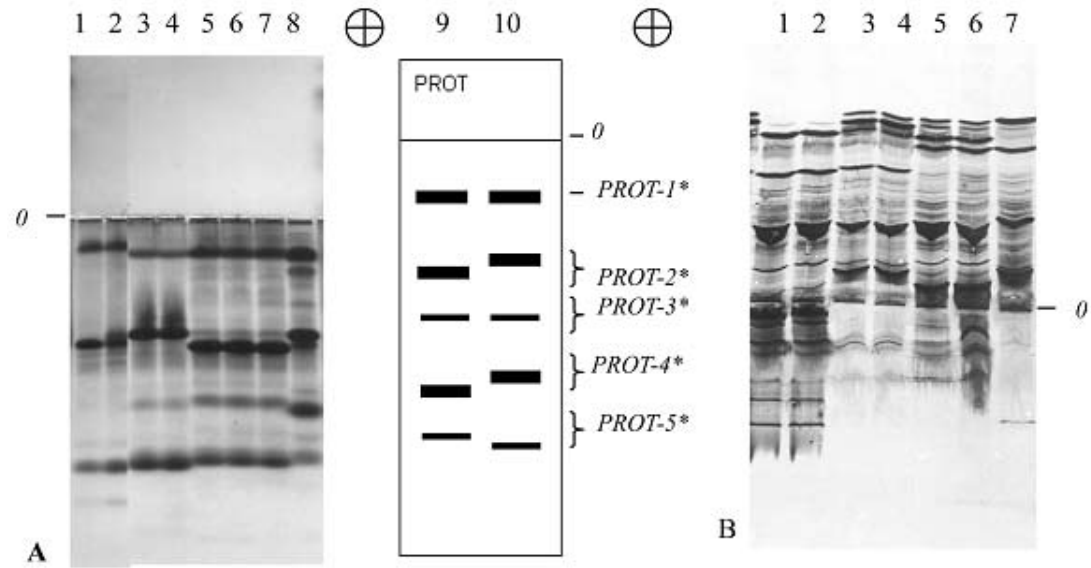

Fig.2. A. Starch gel electrophoresis (general muscle proteins PROT): 1-2-*K. caucasica* (Mandra Lake), 3-4 – *G. niger* (Varna Bay), 5-7 – *G. gymnotrachelus* (Kamchiya River), 8 – *N.* (*Apollonia*) *melanostomus* (Varna Bay), 9 – *K. caucasica* and 10 – *K. longecaudata* (scheme). **B.** Isoelectric focusing (IEF) on ultrathin polyacrilamide Servalyte gel plate with pH range 3-10: 1-2 *N.* (*Apollonia*) *fluviatilis* (Varna Bay), 3-4 –*N. syrman* (Dniester River), 5-6 – *N.* (*Apollonia*) *melanostomus* (Varna Bay) and 7 – *G. ophiochephalus* (Varna Lake)

A single natural hybrid of *N.* (*Apollonia*) *fluviatilis* with *N.* (*Apollonia*) *melanostomus* in the Black Sea was described by Pinchuk (1970) on the basis of morphological analysis. Since hybridization is a more common phenomenon among species with a panspermic way of reproduction, such as cyprinids or loaches (Economidis, Sinis 1988, Choleva *et al.* 2008), hybridization among gobies should happen only accidentally, considering that this group exhibits parental care toward the eggs. Reproductive isolation between closely related species is thus present and leads to low hybridization rates.

Species specific electrophoretical patterns on esterases (*EST-1**, *EST-2**, *EST-3** and *EST-4** loci) of *K. longecaudata* in comparison with *K. caucasica* were also observed (Fig. 5). Genetic distance (D_{Nei} =1.10) and time of divergence (t_{Nei} = 5 500 000 years) between *K. longecaudata* and *K. cau-*

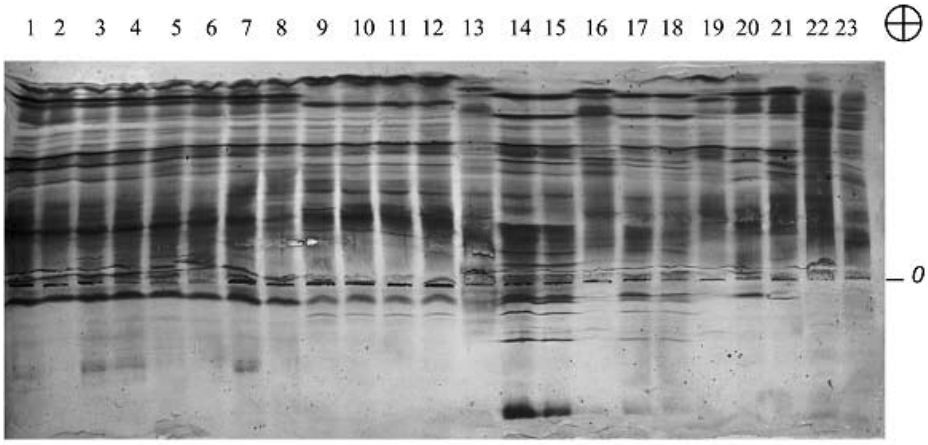

Fig.3. Isoelectric focusing (IEF) on thin polyacrilamide Ampholine gel plate with pH range 3-10: 1-2 – *N.* (*Apollonia*) *melanostomus* – Shabla Lake, 3 – *N.* (*Apollonia*) *melanostomus* – Durankulak Lake, 4 – *N.* (*Apollonia*) *melanostomus* – Belene (Danube River), 5 – *N.* (*Apollonia*) *melanostomus* – Varna Bay, 6 – *N.* (*Apollonia*) *melanostomus* – Shkorpilovtsi, 7-8 – *N.* (*Apollonia*) *melanostomus* – Tutrakan (Danube River), 9 и 11 – *G. niger*, Tyulenovo, 10 and 12 – *G. niger* – Shkorpilovtsi, 13 и 16 – *P. marmoratus* – Perla Bay , 14-15 and 17-18 – *N.* (*Apollonia*) *fluviatilis* – Belene (Danube River), 19 – *M. batrachocephalus* (Tyulenovo), 20- *N.* (*Apollonia*) *melanostomus* (Varna Bay), 21 – *N. gymnotrachelus* (Kamchiya River), 22-23 – *K. caucasica* (Fandaklijska River)

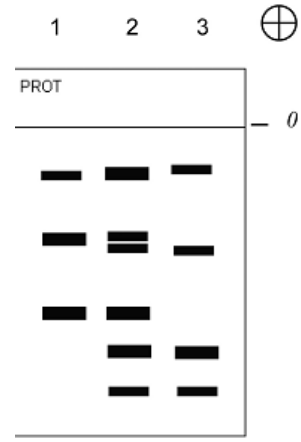

Fig. 4. Scheme on PROT (starch gel): 1- *N.* (*Apollonia*) *fluviatilis*, 3- *N. syrman* and 2- hybrid spectra between *N.* (*Apollonia*) *fluviatilis* and *N. syrman*

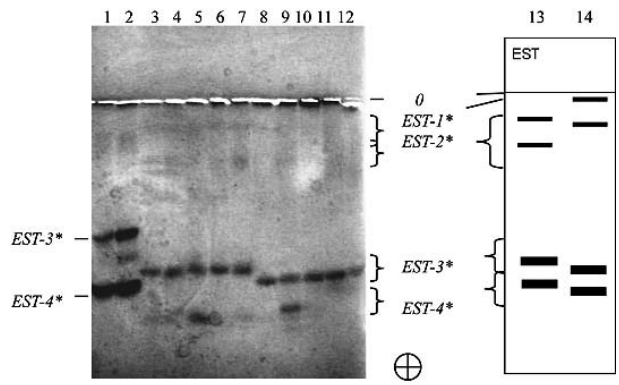

Fig.5. Electrophoregrams of esterases (EST) on starch gel: 1-2 – *N.* (*Apollonia*) *melanostomus* (Varna Bay); 3-7 – *P. marmoratus* (Perla Bay); 8-13 – *K. caucasica* (Fandaklijska River) and 14 – *K. longecaudata* (Shabla Lake), (13-14 – scheme), *EST-1** and *EST-2** – loci with enzyme activity, 0- origin

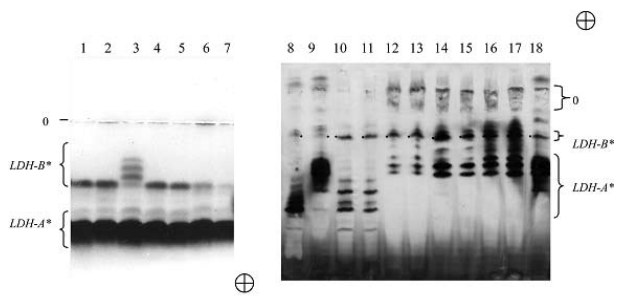

Fig. 6. Zymograms of lactate dehydrogenase on starch gel: 1-3 – *G. niger*, 4-7 – *G. ophiocephalus*. Isoelectric focusing of LDH on thin polyacrilamide Ampholine gel plate, 8 – *N.* (*Apollonia*) *fluviatilis* (Varna Bay), 9 – *N.* (*Apollonia*) *melanostomus* (Varna Bay) and 18 – *N. platyrostris* (muscle tissue), 10-11 – *N.* (*Apollonia*) *fluviatilis* (Varna Bay), 12-13 – *N.* (*Apollonia*) *melanostomus* (Varna Bay), 14-15 – *N. platyrostris* (Black Sea, Odessa) and 16-17. *M. batrachocephalus*, Varna Bay (heart tissue), 0 – origin

casica give evidence for the existence of these two well diverged species in one genus. This enzymic complex showed a high level of polymorphism and can be used as a protein biomarker for the assessment of the population structure of *K. caucasica*. Polymorphism on *EST-4** with null allele locus was found in *P. marmoratus* and *K. caucasica*. Two allelic polymorphisms on this locus were observed in *N.* (*Apollonia*) *melanostomus* (Shabla and Durankulak lakes) and *G. niger* (Table 2). *EST-3** polymorphism in *N.* (*Apollonia*) *melanostomus* (Danube River) and *G. niger* (Varna Lake) was also observed.

Two lactate dehydrogenase loci (*LDH-A** and *LDH-B** loci) were visualized for all the examined species. According to Dobrovolov (1996), species belonging to the same genus have equal *LDH-B** position. The grass goby, *Gobius ophiocephalus,* had *LDH-B** patterns equal to those of other species of the genus *Gobius* (Fig. 6) and should also belong to this genus. Sorice, Caputo (1999) proved that the genus *Zosterisessor* should be absorbed into *Gobius* according to genetic data. Krjizhanovski, Pchelina

Table 2. Estimated allele frequencies of polymorphic loci on esterases and malate dehydrogenase in *G. niger* (Varna Bay)

Locus	Allele	*G. niger*
EST-3*	a* o*	0.580 0.420
EST-4*	a* b*	0.536 0.464
MDH-1*	a* b* c*	0.435 0.558 0.007
LDH-B*	b* b^{1*}	0.697 0.303
MEP-1*	a* b* c*	0.036 0.703 0.261
MEP-2*	a* o*	0.448 0.552

(1941) noted that *Z. ophiocephalus* belonged to the genus *Gobius* and *Zosterisessor* is a synonym. Vassiljeva (2007) also considered that the grass goby is very close to other *Gobius* spp. and, according to morphological and karyological data, the correct name of this species should be *Gobius ophiocephalus*. Our data confirm this assumption, since *LDH-B** spectra of *G. ophiocephalus* and *G. niger* are of the same electrophoretical mobility (Fig. 6).

The samples of *N. fluviatilis* and *N. melanostomus* have the common position of the *LDH-B** fractions, which differ from those of all other *Neogobius* spp. The differences in *LDH-B** locus can be seen in the starch gel as well as in the IEF gel plate (muscle and heart tissue) (Fig. 6), and thus the hypothesis that these two species should be included in the genus *Apollonia* has been confirmed. Phylogenetic results based on the analyses of the mitochondrial cyt b gene indicated that the genus *Neogobius* is paraphyletic and that the subgenus *Apollonia* should therefore be elevated to the level of genus, containing *Apollonia melanostomus* and *A. fluviatilis* (Stepien, Tumeo 2006).

Dobrovolov (1996) assumed that *Neogobius batrachocephalus* should not belong to the genus *Mesogobius,* taking into account the same electrophoretic mobility of the *LDH-B** locus of all *Neogobius* spp. (including also *M. batrachocephalus*) (Fig. 6). Polymorphism regarding the *LDH-B** locus was found only in *G. niger* from Varna Lake (Table 2, Fig. 6).

The genus-specific *LDH-B** spectra of *Pomatoschistus marmoratus* and *K. caucasica* also

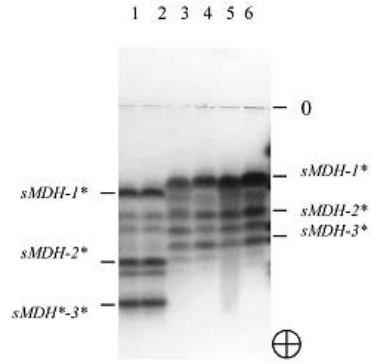

Fig. 7. Starch gel zymogram of malate dehydrogendase (MDH) on starch gel: 1-2 – *G. ophiocephalus*, 3-6 – *G. niger*, 0 – *origin*

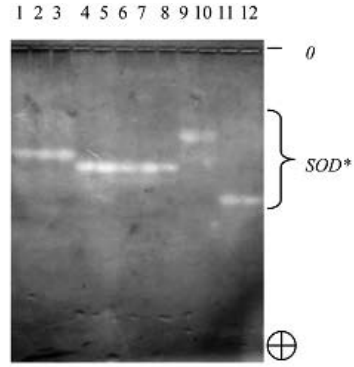

Fig. 8. Electrophoregram of superoxidedismutase (SOD) on starch gel: 1-3 – *G. niger*, 4-8 – *Pomatoschistus marmoratus*, 9-10 – *G. ophiochephalus*, 11-12 – *N.* (*Apollonia*) *melanostomus*, 0 – *origin*

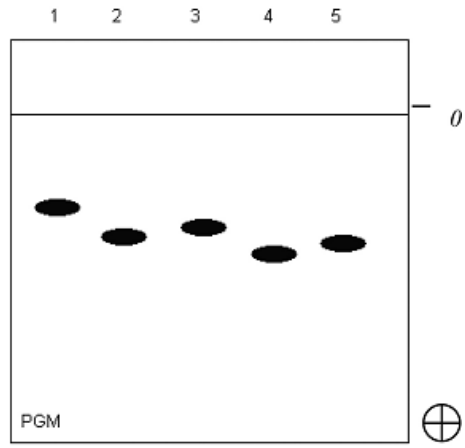

Fig. 9. Scheme of phosphoglucomutase (PGM) on starch gel: 1 – *M. batrachocephalus*, 2 – *N.* (*Apollonia*) *melanostomus*, 3 – *N.* (*Apollonia*) *fluviatilis*, 4 – *G. ophiocephalus* and 5 – *G. niger*

have different electrophoretic mobility. *LDH-A** locus was monomorphic and species specific.

Three malate dehydrogenase loci were monomorphic in most of the studied species, with the ex-

Table 3. Polymorphism of 12 gobiid species from various Bulgarian water basins in regard to 5 protein and 24 enzymic loci. 1 – lack of polymorphism, N>1 – number of alleles in locus, * – null allele, – - absence of given locus expression for given species, 0 – the species is not analysed in terms of given locus.

Locus		Species	*Knipowitschia caucasica*	*Knipowitschia longecaudata*	Pomatoschistus marmoratus	Gobius niger	Gobius ophiocephalus	Proterorinus marmoratus	Neogobius melanostomus	Neogobius fluviatilis	Neogobius gymnotrachelus	Neogobius batrachocephalus	N. syrman	N. platyrostris
1	*PROT-1**		1	1	1	1	1	1	1	1	1	1	1	1
2	*PROT-2**		1	1	1	1	1	1	1	1	1	1	1	1
3	*PROT-3**		1	1	1	1	1	1	1	1	1	1	1	1
4	*PROT-4**		1	1	1	1	1	1	1	1	1	1	1	1
5	*PROT-5**		1	1	1	1	1	1	1	1	1	1	2*	-
6	*EST-1**		1	1	1	1	1	1	1	1	1	1	1	2
7	*EST-2**		1	1	1	1	1	1	1	1	1	1	1	2
8	*EST-3**		1	1	1	2*	1	1	2	1	1	1	1	1
9	*EST-4**		2*	1	2*	2	1	1	2	1	1	1	1	1
10	*LDH-A**		1	1	1	1	1	1	1	1	1	1	1	1
11	LDH-B*		1	1	1	2	1	1	1	1	1	1	1	1
12	*MDH-1**		1	1	1	1	1	1	1	1	1	1	1	1
13	*MDH-2**		1	1	1	1	1	1	2	1	1	1	1	1
14	*MDH-3**		1	1	1	1	1	1	1	1	1	1	1	1
15	*sMEP-1**		1	1	1	3	1	1	1	1	1	1	1	1
16	*sMEP-2**		1	1	1	2*	1	1	2	1	1	1	1	1
17	*mMEP**		1	1	1	1	1	1	1	1	1	1	1	1
18	*SOD**		1	1	1	1	1	1	1	1	1	1	1	1
19	*PGM**		1	1	1	1	1	1	1	1	1	1	1	1
20	*ADH**		1	1	1	1	1	1	1	1	1	1	0	0
21	*G6PDH-1**		1	1	1	1	1	1	1	1	1	1	0	0
22	*G6PDH-2**		1	1	1	1	1	1	1	1	1	1	0	0
23	*GPI-1**		1	1	1	1	1	1	1	1	1	1	1	1
24	*GPI-2**		1	1	1	1	1	1	1	1	1	1	1	1
25	*GPI-3**		1	1	1	1	1	1	1	1	1	1	1	1
26	*GPI-4**		1	1	1	1	1	1	1	1	1	1	1	1
27	*GLUDH**		1	1	1	1	1	1	1	1	1	1	0	0
28	*G3PDH-1**		1	1	1	1	1	1	1	1	1	1	0	0
29	*G3PDH-2**		1	1	1	1	1	1	1	1	1	1	0	0
Total number of analysed loci			29	29	29	29	29	29	29	29	29	29	23	22
Heterozygotic loci			1	0	1	5	0	0	4	0	0	0	1	2
Heterozygosity in % of the analysed loci			3.45	0	3.45	17.24	3.45	3.45	13.79	0	3.45	3.45	4.35	9.09

ception of *G. ophiocephalus*, *N.* (*Apollonia*) *fluviatilis* and *G. niger*. The polymorphic locus (*MDH-1**) of *G. niger* is controlled by three co-dominant alleles (Table 2).

Regarding the malic enzyme, three loci were observed (*sMEP-1*, sMEP-2** and *mMEP**). Electrophoretical spectra of the analysed species are specific and appeared to be mostly monomorphic. Polymorphism on *sMEP-1** and *MEP-2** was found only in *G. niger* and only on *MEP-2** in *N.* (*Apollonia*) *melanostomus*.

The remaining species specific enzyme sys-

tems which have been analysed appear to be monomorphic: alcoholdehydrogenasae (ADH) – one locus, glycerol 3-phosphate dehydrogenase (G3PDH) – two loci, glucosephosphate isomerase (GPI) – four loci, glutamate dehydrogenase (GLUDH) – one locus, glucose-6-phosphate dehydrogenase (G-6PDH) – two loci, malatedehydrogenase (MDH) – three loci, phosphoglucomutase (PGM) – one locus and superoxiddismutase (SOD) – one locus (Figs. 7, 8 and 9, Table 3).

As noted in Table 3, most heterozygotic loci were found in *G. niger* and *N.* (*Apollonia*) *melanostomus*, the species which were most abundant in commercial catches in the Bulgarian Black Sea sector (Vassilev *et al.* 2011). *N.* (*Apollonia*) *melanostomus* is highly invasive Ponto-Caspian species, while *G. niger* is a Mediterranean immigrant in the Black Sea. Another species, *P. marmoratus,* with high invasive potential, showed a remarkably low rate of polymorphic loci, whereas a more range restricted species, such as *N. platyrostris*, was comparatively variable in relation to other examined species. On the basis of the obtained data, we assume that invasiveness is not always connected with genetic diversity.

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