ECOLOGIA BALKANICA

2014, Vol. 6, Issue 1 June 2014 pp. 37-44

Genetic Differentiation between Mullus barbatus from the Western Part of the Black Sea and Mullus surmuletus (Pisces, Mullidae) from the Mediterranean Sea

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Abstract. Genetic divergence and phylogenetic relationship of two species *Mullus barbatus* from the western part of Black Sea (Varna) and *M. surmuletus* from the Mediterranean Sea (Thessaloniki) were investigated using the electrophoretic data from enzymatic systems, codifying for 15 putative loci, and the patterns of general muscle proteins (PROT) coded from nine loci. Several loci *PROT-4*, PROT-5** and *PROT-8** as well as two *mMDH* and two *sMDH* loci, and *LDH-A** showed different electrophoretic patterns among species and can be used as species-specific markers. Only one esterase locus (*EST-9**) was found to be polymorphic for both species. The remaining enzymes and proteins were monomorphic. In this study for the first time existence of hybrids between two species were reported. Hybrids were registered in the Mediterranean Sea (Thessaloniki) as well in the northeastern part of Black Sea (Balshoj Utrish) using electrophoresis and isoelectric focusing methods. Genetic distance D_{Nei} (0.526) and time of divergence (t_{Nei} = 3 215 000 years) between *M. barbatus* (Varna Bay) and *M. surmuletus* (Thessaloniki) give evidence for existence of these two well diverged species in one genus.

Key words: genetic divergence, phylogenetic relationship, *Mullus barbatus, Mullus surmuletus,* Black Sea, Mediterranean Sea.

Introduction

The genus *Mullus* is represented by two species - *M. barbatus* and *Mullus surmuletus*.

M. barbatus L. 1758 is distributed throughout the Mediterranean Sea, as well as in the eastern Atlantic, from the British Islands in the north to Senegal in the south (HUREAU, 1986, cited after TURAN, 2006). *Mullus barbatus* in the Black Sea is taxonomically classified as a subspecies *M. barbatus ponticus* Essipov, 1927 (KARAPETKOVA & ZHIVKOV, 2006; TURAN, 2006).

KESKIN & CAN (2009) and VASILJEVA (2012) on the base of molecular, morphological and karyological data have not verified the existence of *M .barbatus ponticus* as a subspecies.

M. surmuletus L., 1758 is distributed throughout the Mediterranean Sea, in the Atlantic, from Norway to the Canary Islands, in the Black Sea and in the northwestern coasts of Africa. VASILJEVA (2007) pointed that this species is registered only along Turkish coast of the Black Sea.

Some studies analysed the genetic variation in *M. barbatus* and *Mullus surmuletus*, identifying diagnostic loci between the two species using allozymes (BASAGLIA & CALLEGARINI, 1988; CAMMARATA *et al*., 1991; MAMURIS *et al*., 1998, 1999). ARCULEO *et al*. 1999; TURAN, 2006). MAMURIS *et al*. (1998) suggested that allozyme analysis provide important information of the genetic structure of the red mullet to ensure sustainable management of this species. MAMURIS *et al*. (2001) and APOSTOLIDIS *et al*. (2001) investigated genetic structure of *M. barbatus* and *Mullus surmuletus* in the Mediterranean Sea, by means of RFLP analysis of PCR-amplified mitochondrial DNA.

According to VASILJEVA (2012) the level of genetic divergence between Mediterranean and Black Sea red mullets is not defined.

The main **goals** of this study were to find diagnostic loci between *M. barbatus* from Bulgarian Black Sea coast and *M. surmuletus* from Mediterranean, to assign the genetic distances between them as well as to find interspecies hybrids.

Material and Methods

54 fish *M. barbatus* from the Black Sea (Varna Bay), 10 fishes from Bolshoj Utrish (Russia) and 15 fish *Mullus surmuletus* from Mediterranean Sea (Thessaloniki) were collected from 1993 -2010 (Fig.1.)

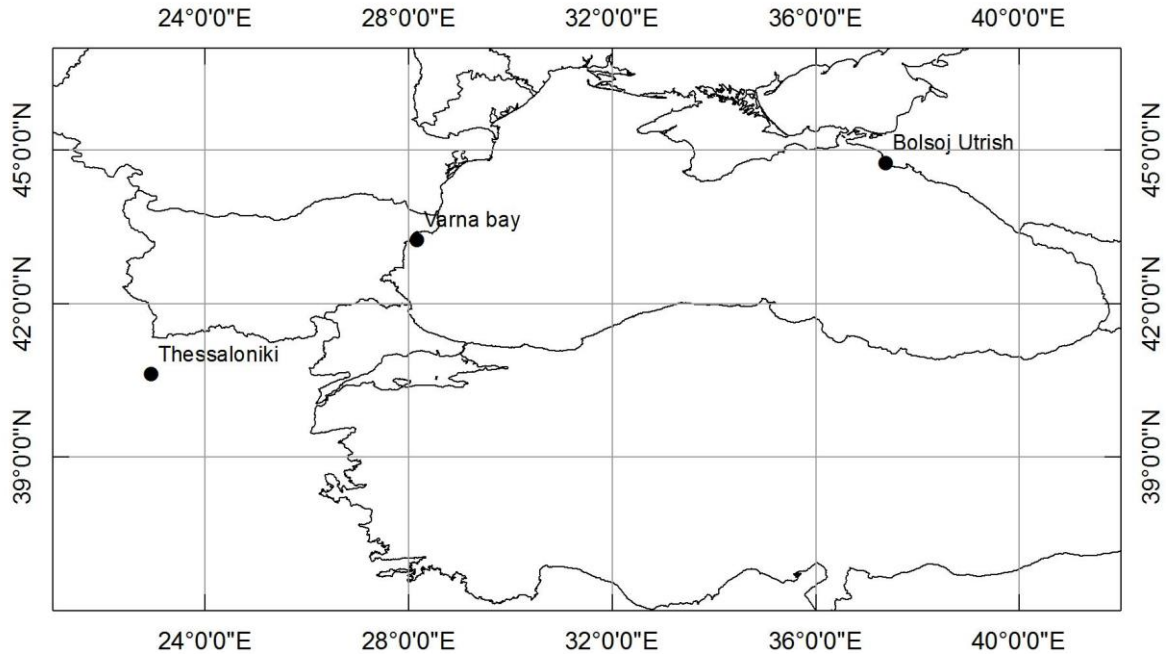

Fig.1. Map of sampling locations for Mullidae species.

For the analysis of the enzymes and non-enzyme protein systems, a homogenate of white dorsal muscle 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 ultra-thin 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 and Prasad (1970). Buffer systems of DOBROVOLOV (1976) and CLAYTON & GEE (1969) were used for the electrophoresis. The following enzymatic systems were studied: esterase (EC 3.1.1.1 - EST), lactate dehydrogenase (EC 1.1.1.27 - LDH) and malate dehydrogenase (EC 1.1.1.37 – MDH).The nomenclature of mentioned loci and alleles followed essentially the recommendation of SHAKLEE *et al*. (1990). Gene frequencies of the polymorphic loci were calculated using

the Hardy-Weinberg equilibrium. Calculation of indices of genetic similarity and genetic distance was performed according to NEI (1972).

Results and Discussion

General muscle proteins (PROT) **-** The general muscle protein fractions (PROT) on isoelectric focusing (IEF) on ultra thin gel plate as well as on starch gel electrophoresis (Fig. 2 and 3) of the examined mullid species demonstrated differences on the species level. Nine loci on general muscle proteins were analyzed (Fig.3) and the difference between the species were presented. The data received, support the CAMMARATA *et al*. (1991) evidence for existence of species-specific patterns on general muscle proteins on the two species compared. The *M. barbatus* samples, caught at different localities of the Black Sea (Varna and Boljshoj Utrish) have equal electrophoretic patterns (Fig. 2 and 3). On Figure 2 one of the samples (N2) showed spectra, typical for the hybrids between different species. Obviously in the Mediterranean Sea the hybrids between *M. barbatus* and *Mullus surmuletus* is registered. Hybrids between two species were found also in Balshoj Utrish (north-eastern part of the Black Sea, Fig.3, N13-15). All analyzed samples from this area showed the same hybrid spectra.

In the Black Sea three allelic type of inheriting of *EST-9** locus (Fig.4, Table 1), while in Mediterranean two allelic polymorphism on this locus were observed. Other analyzed esterase loci were monomorphic. Species specific electrophoretical spectera on *EST-2**, *EST-7**and *EST-8** of *Mullus surmuletus* and *M. barbatus* were were observed (Table 1).

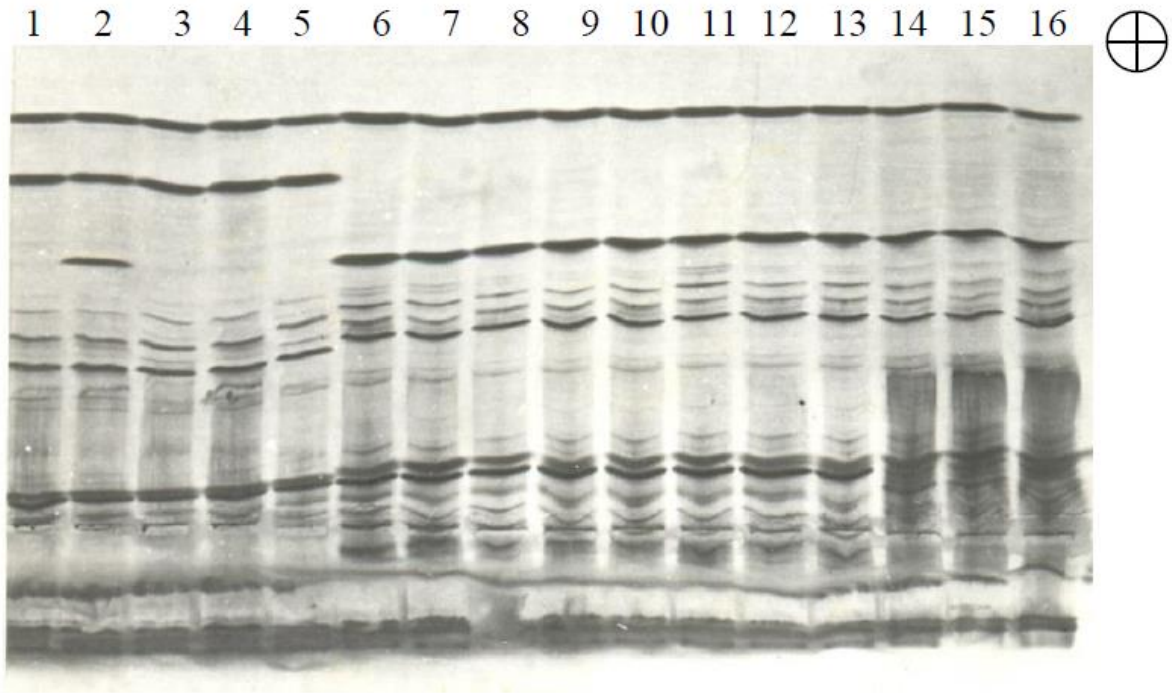

Fig. 2. Isoelectric focusing (IEF) on ultrathin polyacrilamide Ampholine gel plate with pH range 3-10: 1-5 - *Mullus surmuletus*, Mediterranean Sea, 6-13 - *M. barbatus*, Varna Bay, Black Sea, 14-16 *M. barbatus,* Balshoj Utrish, Russia.

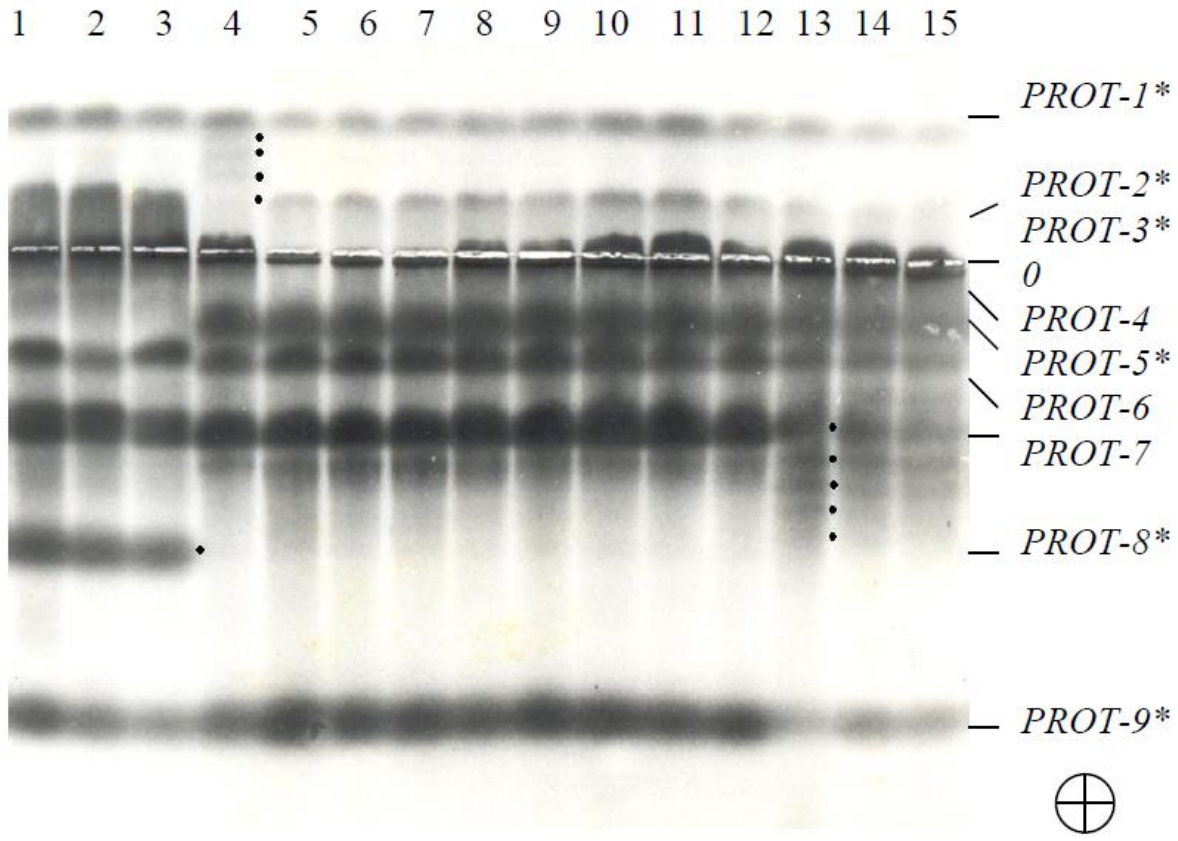

Fig.3. Electrophoregrams on general muscle proteins (PROT) : 1-3 - *Mullus surmuletus*, Mediterranean Sea, 4-12 - *M. barbatus* , Varna Bay, Black Sea, 13-15- *M. barbatus,* Balshoj Utrish, Russia, showed hybrid spectra, marked with dots, 0 – origin.

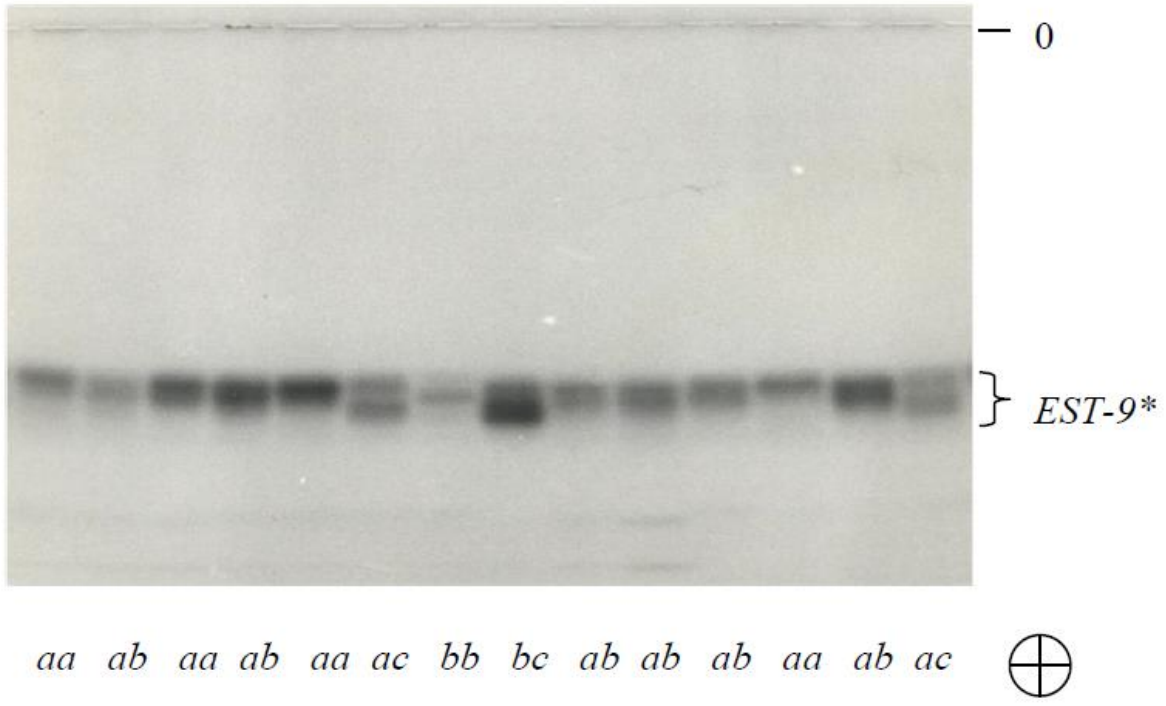

Fig.4. Electrophoregrams of esterases (EST) on starch gel on *Mullus barbatus,* Varna Bay. Polymorphism with three allelic type of co-dominant inheriting was registered. aa, ab and bb – phenotypes. O – origin.

Except esterases, the remained species specific enzyme systems which have been analyzed occurred to be monomorphic (Table1).

Concerning lactate dehydrogenase, two loci (*LDH-A** and *LDH-B**) were visualized on the electrophoregrams. According to DOBROVOLOV (1996) the species, which belong to the same genus have equal *LDH-B** position. The genus specific *LDH-B** spectra of red mulled and striped mulled has also equal electrophoretic mobility. The observed lack of differences at the locus *LDH-B** between two species is consistent with the results obtained by CAMMARATA *et al.* (1991) and MAMURIS *et al.* (1998). *LDH-A** locus was monomorphic with different electrophoretic mobility by two species compared. This locus is species specific (Fig.5).

Four malate dehydrogenase loci were monomorphic in the investigated species. Two *sMDH* loci and two *mMDH* loci were observed with species specific differences on the both species (Fig.6). The samples from Balshoj Utrish showed hybrid spectra on this enzyme system.

The hybrids have fractions of two species analyzed.

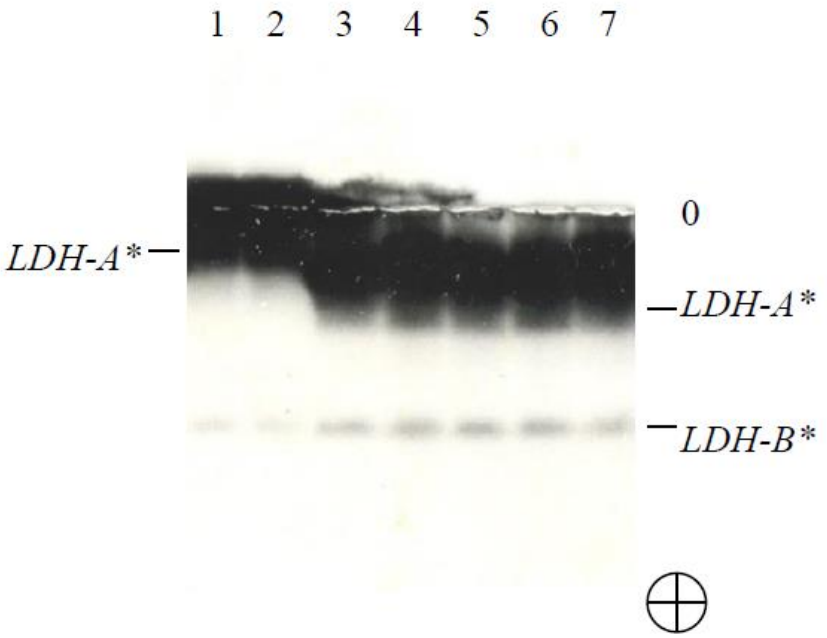

Fig.5. Zymograms of lactate dehydrogenase on starch gel: 1-2 - *Mullus surmuletus*, Mediterranean Sea, 3 – 5 - *M. barbatus*, Varna Bay, Black Sea, 6-7 *M. barbatus,* Balshoj Utrish, Russia

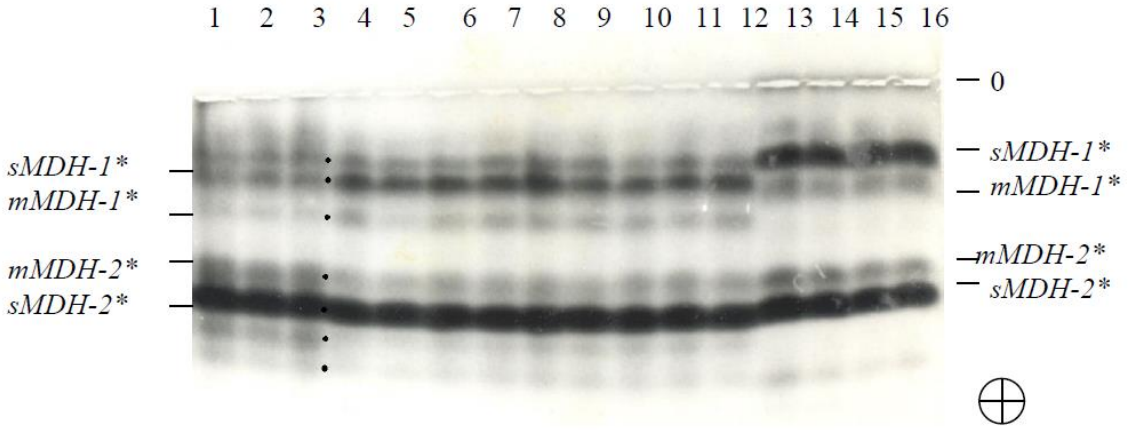

Fig.6. Zymogram of malate dehydrogendase (MDH) on starch gel:1-3 *M. barbatus,* Balshoj Utrish, Russia (hybrid spectrum was marked with dots), 4-12 - *M. barbatus* , Varna Bay, Black Sea, 13-16 - *Mullus surmuletus*, Mediterranean Sea, 0-origin.

Table 1. Genetic distance (D_{Nei}) between species *M. barbatus* (Black Sea, Varna Bay) and *Mullus surmuletus* (Mediterranean Sea, Thessaloniki), calculated on the base of 9 protein and 15 enzymic loci.

Species Alele	*M. barbatus* Black Sea	*Mullus surmuletus* Mediterranean Sea	D_{Nei}
*EST-1**	1	1	1
*EST-2**	0	1	0
*EST-3**	1	1	1
*EST-4**	1	1	1
*EST-5**	1	1	1
*EST-6**	1	1	1
*EST-7**	0	1	0
*EST-8**	1	0	0
*EST-9**	a-0.544 b-0.324 c-0.132	a-0.125 b-0.875 c-0	0.615
*PROT-1**	1	1	1
*PROT-2**	1	1	1
*PROT-3**	1	1	1
*PROT-4**	0	1	0
*PROT-5**	1	0	0
*PROT-6**	1	1	1
*PROT-7**	1	1	1
*PROT-8**	0	1	0
*PROT-9**	1	1	1
*LDH-A**	1	0	0
*LDH-B**	1	1	1
*sMDH-1**	0	1	0
*sMDH-2**	1	0	0
*mMDH-1**	0	1	0
*mMDH-2**	1	0	0
D_{Nei}			0.526

Genetic distance D_{Nei} (0.526) and time of divergence (t_{Nei} = 3 215 000 years) between *M. barbatus* from the Black Sea and *M. surmuletus* from Mediterranean, calculated on the base of 24 analyzed loci, give evidence for existence of these two well divergated species in one genus.

The Nei's genetic distance presented is more close to this (D=0.329), calculated from MAMURIS *et al*. (1998) between the two species *M. barbatus* and *M. surmuletus* in Mediterranean Sea. The higher genetic distance calculated by us could be result form the comparison of two species, inhabited two basins (Black Sea and Mediterranean Sea). We did not support CAMMARATA *et al*. (1991) opinion for high similarity between the two *Mullus* species (D =0.068).

Allozyme data of analyzed samples from Balshoj Utrish showed that they are 100% hybrids between *M. barbatus* and *M. surmuletus* and prove the existence of the *M. surmuletus* species along the Russia coast.

One morphological parameter - standard length (SL) measured for the *M. surmuletus* from Thessaloniki (Mediterranean Sea) varied from 16 to 20 cm, for the *M. barbatus* from Varna Bay (Black Sea) – between 10.7 and 15.6, while of the hybrid samples from Bolshoj Utrish have intermediate values from 14.5 to 17.5cm.

Conclusions

General muscle proteins and enzymes analysed could be used as species specific markers.

The genetic distance, calculated on the basis of alloszymes between *M. barbatus* and *M. surmuletus* (D=0.526) and time of divergence (t_{Nei} = 3 215 000 years) give evidence for existence of two well divergated species in one Genus.

For the first time hybrids between *M. barbatus* and *M. surmuletus* in Mediterranean and Black Sea (Boljshoj Utrish, Russia) were registered using two electrophoretical methods.

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Received: 27.03.2014
Accepted: 30.05.2014