

## Population – genetic structure on European anchovy (*Engraulis encrasicolus*, Linnaeus, 1758) (Osteichthyes: Engraulidae) from Mediterranean Basin and Atlantic Ocean

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*Muscle proteins of the European anchovy *Engraulis encrasicolus* L. were analyzed using starch gel electrophoresis and isoelectric focusing on thin polyacrylamide ampholine gel. Twenty-two protein loci were analyzed and polymorphism was found in six of them. Based on genetic-biochemical data, we hypothesize that there are two anchovy subspecies, European and African. The former inhabits the Atlantic Ocean, the Mediterranean coast of Europe, and the Aegean, Marmora, Black, and Azov Seas. The latter is found in the Cape Blank region of the Atlantic Ocean and, probably, along the northwestern part of the African coast. The Aegean anchovy consists of hybrid populations, resulting from introgressive hybridization between the European and the African populations. No evidence for subspecies differentiation between the populations from the European coast of the Mediterranean and the Atlantic Ocean was found. Genetic distances between the Azov and Black Sea populations show that the former probably entered the Black Sea during the Karangad period and the latter during the last connection of the Black Sea to the Mediterranean. The genetic distance between the Black Sea anchovy and the Azov anchovy shows that they could be specified as two different populations. Probably some earlier subspecies differences disappeared as a result of introgressive hybridization.*

**Key words:** anchovy, population structure, genetic distance, Mediterranean Basin, Atlantic Ocean

### INTRODUCTION

The European anchovy, *Engraulis encrasicolus* L., is a shoaling clupeoid that is distributed along the eastern Atlantic coast from Scandinavia to west Africa. It is also found in the Mediterranean, Black, and Azov Seas (WHITEHEAD *et al.*, 1988). FAGE (1911, 1920) subdivided the European anchovy into two races, the Atlantic and the Mediterranean, and further subdivided the Atlantic anchovy into northern

and southern groups, and the Mediterranean anchovy into western and eastern groups. The latter group included anchovies from the Aegean, Marmora, Black, and Azov Seas.

According to ALEXANDROV (1927), the Black Sea anchovy inhabited the western part of the Black Sea while the Azov anchovy inhabited the eastern part of the Black Sea, entering the Azov Sea for reproduction and nurture. POUSSANOV (1936) combined the Black Sea and the Mediterranean anchovy into one race and

considered the Azov anchovy another species, *Engraulis maeoticus*.

Based on immunological analyses, ALTUCHOV (1974) found differences between the Black Sea and the Azov anchovies. SHEVCHENKO (1980) considered the Azov anchovy a relict form from the Karangatian period and the Black Sea anchovy a subsequent immigrant from the contemporary Mediterranean period. KALNINA & KALNIN (1984), KALNIN *et al.* (1984), KALNIN, & KALNINA (1985) examined introgressive hybridization, population structure, biochemical polymorphism, genetic differentiation, and reproductive isolation of two anchovy races in the Black and Azov Seas using biochemical-genetic methods.

DOBROVOLOV (1976, 1978, 1987, 1992) and DOBROVOLOV *et al.* (1980) studied the phylogenetic relationship between *E. encrasicolus* from the Atlantic Ocean and from the Mediterranean Basin on the basis of allozyme analyses. GARCIA *et al.* (1994, cited after BEMBO *et al.*, 1995) reported an absence of genetic structuring of anchovy inhabiting the western Mediterranean after an allozyme study. SPANAKIS *et al.* (1989) distinguished anchovy stocks from the Ionian and Aegean Seas by morphometric and allozyme analyses.

MtDNA markers can be used for species identification and detection of intraspecific variation in *E. encrasicolus* stocks (CHOW *et al.*,

1993; CRONIN *et al.*, 1993; MARGOULAS & ZOUROS, 1993; CHAPMAN *et al.*, 1994, cited after BEMBO *et al.*, 1995). BEMBO *et al.* (1995) studied the mtDNA variation in *E. encrasicolus* in the northwestern Mediterranean. Their DNA data were in accordance with their earlier allozyme and meristic results. MARGOULAS & ZOUROS (1993) and MARGOULAS *et al.* (1996) studied mtDNA variation in *E. encrasicolus* and found two main anchovy mtDNA phylads in the Mediterranean: phylad A dominated in the Black and Aegean Seas while phylad B was more numerous in western waters.

The present paper aims to summarize long-term genetic-biochemical data for clarifying the intraspecific divergence of anchovy from different populations: the Black Sea (western and eastern part), the Azov, Marmora, Aegean, Adriatic, and Mediterranean Seas, Canary Islands and Cape Blank (Africa) using two electrophoretic techniques.

## MATERIAL AND METHODS

### Sampling

A total of 3 520 specimens of anchovy (*Engraulis encrasicolus*) were collected from 11 localities during 1973-2004 (Table 1, Fig.1). Samples for electrophoretic analysis were captured by fishing vessels and trap-nets. The

Table 1. *Engraulis encrasicolus* samples collected during 1973-2004

Region No.	Year	Region	No. specimens
1	1973	Atlantic Ocean (Cape Blank)	84
2	1980	Atlantic Ocean (Grand Canary)	98
3	1994	Mediterranean Sea (Valencia)	72
4	1994	Mediterranean Sea (Nice)	32
5	1988	Adriatic Sea (near Venice)	100
6	1978	Aegean Sea (near Piraeus)	41
7	1993	Aegean Sea (near Thessalonica)	68
8	1983, 1991	Sea of Marmora (Istanbul)	352
9	1973, 1975-1980, 1982, 1988, 1994, 1997	Black Sea (Bulgarian coast)	1841
10*	1975, 1981, 1989	Sea of Azov	593
11	1989	Black Sea (near Poti)	239

\* Identified as 'Portuguese' in our previous papers



Fig. 1. Sampling locations of anchovy *E. encrasicolus*, collected for electrophoresis study

fish were frozen immediately after catch and brought to the laboratory in portable refrigerators or dry ice at  $-20^{\circ}\text{C}$ . Samples from the Black Sea were frozen in the Institute of Fisheries and Aquaculture (IFA) laboratory in Varna.

#### Starch gel electrophoresis

Proteins were separated by horizontal starch gel electrophoresis according to SMITHIES (1955), modified by DOBROVOLOV (1973). Muscle proteins were stained with Amido Black 10B. Buffer systems of DOBROVOLOV (1976) and CLAYTON & GEE (1969) were used for the electrophoresis.

#### Isoelectric focusing (IEF)

LKB (Stockholm) equipment was used for isoelectric focusing (IEF) on thin polyacrilamide ampholine gel with pH gradients of 3.5-10. Muscle proteins were stained with Coomassie Brilliant Blue R-250. When several forms of the same protein (enzyme) were observed, loci were identified by number, beginning with one for the locus closest to the cathode. Gene frequencies of the polymorphic loci were calculated using the HARDY-WEINBERG equilibrium. Calculations of indices of genetic

similarity and genetic distance were performed according to NEI (1972). Nomenclature for loci and alleles essentially follows the recommendations of SHAKLEE *et al.* (1990).

### RESULTS AND DISCUSSION

Several authors have suggested the existence of anchovy subspecies (races) in the Mediterranean on the basis of variation in morphological characters but the taxonomic status of these divisions remains doubtful (SPANAKIS *et al.*, 1989). Polymorphism of general muscle proteins (PROT) could be used to determinate taxonomic differences in the anchovy population.

Four polymorphic zones were found after analysis of general muscle proteins using starch gel electrophoresis (DOBROVOLOV, 1978, 1992; DOBROVOLOV *et al.*, 1980). Since the second polymorphic zone (*PROT-2\**) covered the first (*PROT-1\**) on the starch gel electrophoregrams, they could not be accurately differentiated and the application of other electrophoretical techniques was necessary. By using isoelectric focusing (IEF) on thin polyacrilamide ampholine gel), six

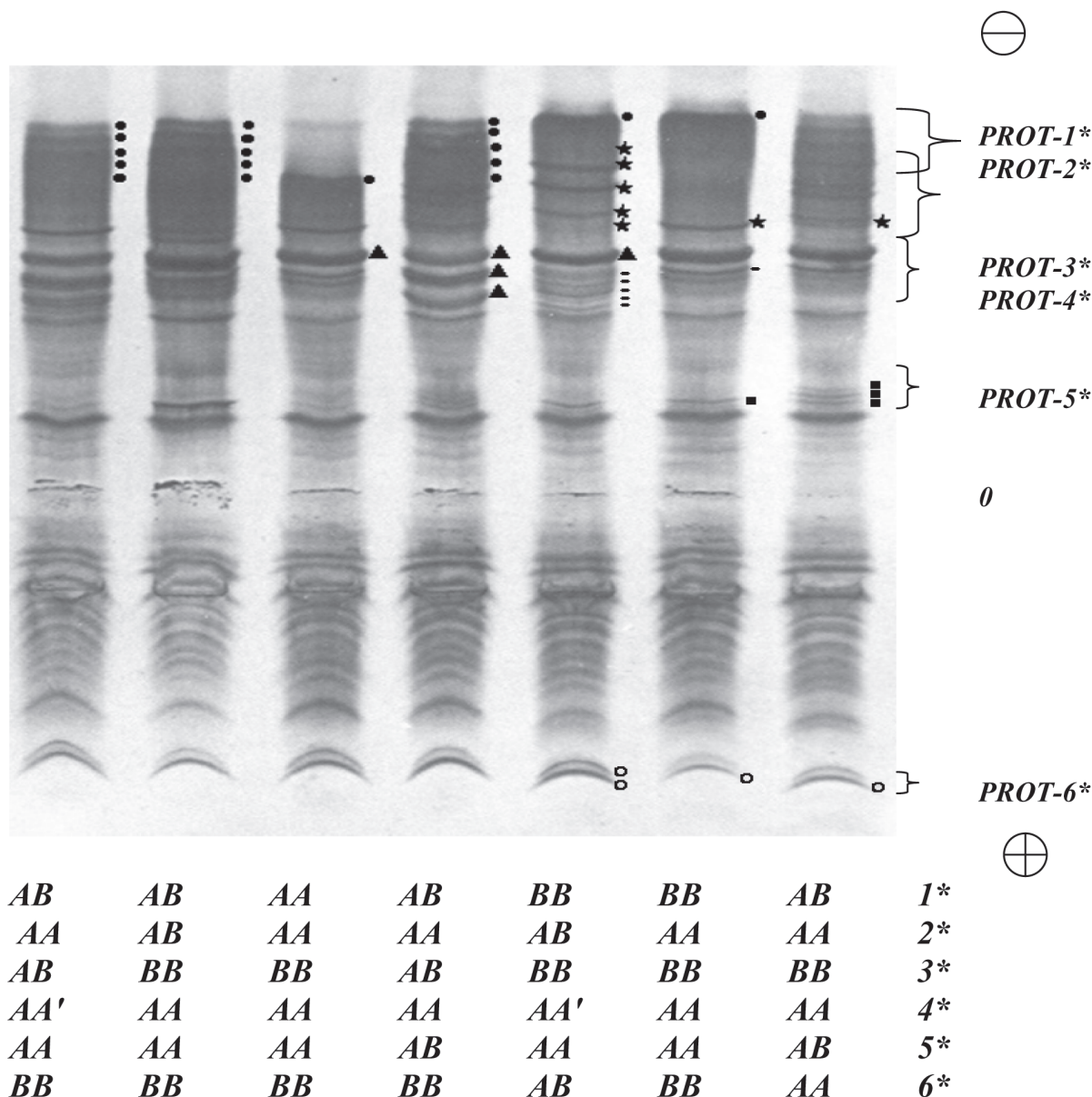


Fig. 2. Isoelectric focusing (IEF) of anchovy muscle proteins on thin polyacrylamide ampholine gel with pH 3.5-10 (1500V, 25mA, 30W, 120 min); *PROT-1\**, *2\**, *3\**, *4\**, *5\**, and *6\** = polymorphic loci; *AA*, *BB*, *AB*, and *AA'* = genotypes; Overlapping polymorphic zones are marked as: • *PROT-1\**, ★ *PROT-2\**, ▲ *PROT-3\**, — *PROT-4\**, ■ *PROT-5\**, ○ *PROT-6\**; *O* = origin

polymorphic zones were found (Figs. 2, 3).

The use of two electrophoretical methods enabled us to obtain more precise information about the above-mentioned polymorphism. The polymorphic zones *PROT-1\**, *2\**, *3\** had lipase activity while most of the monomorphic protein fractions had esterase activity (DOBROVOLOV, 1987). The fourth polymorphic zone had

lactate dehydrogenase expression (*LDH-A\** polymorphism; DOBROVOLOV, 1976, 1978). The other two polymorphic zones, *PROT-5\**, *6\**, were found by using IEF. The observed polymorphism follows the HARDY-WEINBERG equilibrium where  $\chi^2$  is lower than 3.84 (df = 1).  $\chi^2$  digression was found only in the samples from the Aegean Sea (Kavala) where the  $\chi^2$  value

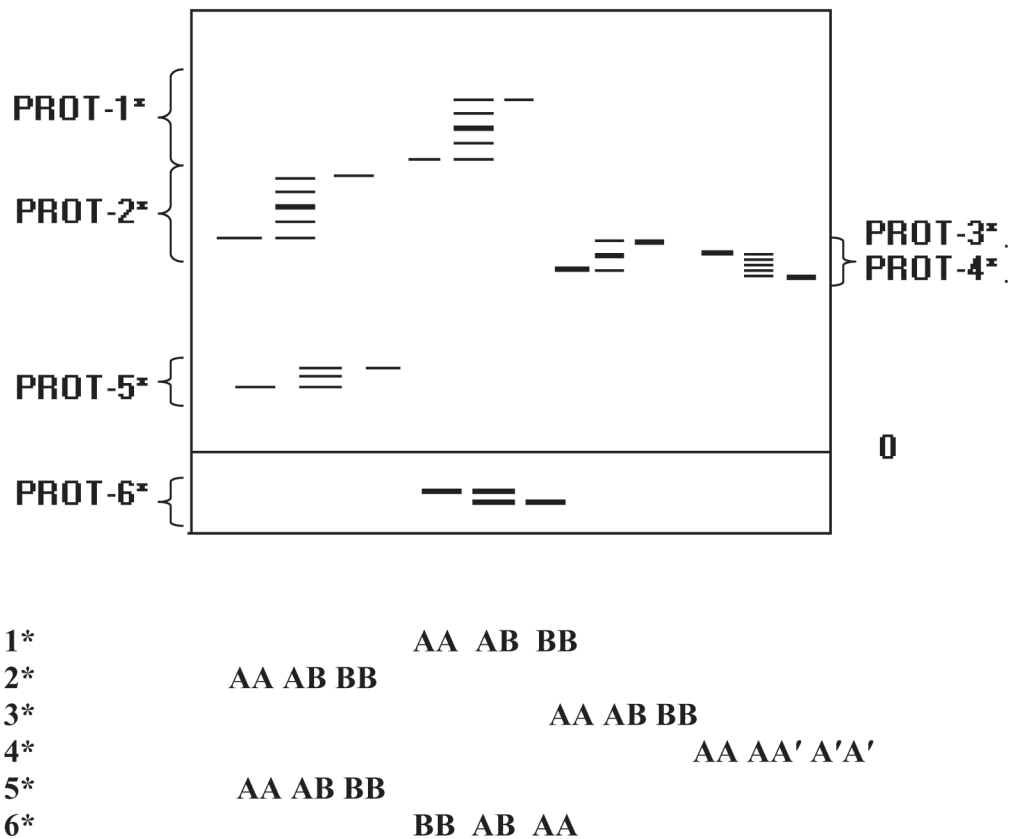


Fig. 3. Scheme of isoelectric focusing (IEF) of general muscle proteins from anchovy on thin polyacrylamide gels with pH 3.5 - 10; PROT-1\*, 2\*, 3\*, 4\*, 5\*, and 6\* = polymorphic loci; AA, BB, AB, AA', and A'A' = genotypes; 0 = origin

was 3.928 in the first polymorphic zone (PROT-1\*;  $p > 0.005$ ). The reason for this high value probably was that samples from two populations were mixed (mechanically). Therefore, these

data are not included in Tables 2 and 3.

The genetic-biochemical analyses show that the Azov anchovy appears sporadically near the Bulgarian coast. The Aegean population stands

Table 2. Estimated allele frequencies of polymorphic loci on general muscle proteins (PROT)<sup>1</sup> in anchovy from 11 investigated regions (locations given in Table 1)

Locus	Allele	Region no.										
		1	2	3	4	5	6	7	8	9	10	11
PROT-1*	a*	0.975	0.977	0.881	0.882	0.634	0.552	0.965	0.953	0.840	0.954	0
	b*	0.025	0.023	0.119	0.118	0.366	0.448	0.035	0.047	0.160	0.046	1
PROT-2*	a*	0.874	0.879	0.961	0.866	0.829	0.546	0.720	0.625	0.639	0.740	0.980
	b*	0.126	0.121	0.039	0.134	0.171	0.454	0.280	0.375	0.361	0.260	0.020
PROT-3*	a*	0.721	0.728	0.607	0.768	0.817	0.877	0.685	0.859	0.813	0.694	0.401
	b*	0.279	0.272	0.393	0.232	0.183	0.123	0.315	0.141	0.187	0.306	0.599
PROT-4*	a*	0.915	0.928	0.902	0.875	0.707	0.750	0.705	0.803	0.771	0.735	0.325
	b*	0.085	0.072	0.098	0.125	0.293	0.250	0.295	0.187	0.229	0.265	0.625

<sup>1</sup> Eighteen fractions with common electrophoretical mobility were observed on the PROT electrophoregrams. Polymorphic zones PROT-5\* and PROT-6\* were decoded only in the last few years (in the Black Azov and Adriatic Seas) and are not included in calculations of  $I_{Nei}$  in Table 3



Table 3. Genetic distance ( $D_{Nei}$ ; above asterisks) and genetic identity ( $I_{Nei}$ ; below asterisks) based on 22 loci for European anchovy (*Engraulis encrasicolus*) from 11 populations (locations given in Table 1)

Region no.	1	2	3	4	5	6	7	8	9	10	11
1	*	0.0000	0.0015	0.0005	0.0088	0.0192	0.0034	0.0051	0.0050	0.0024	0.0740
2	1.0000	*	0.0017	0.0005	0.0080	0.0190	0.0040	0.0052	0.0051	0.0027	0.0752
3	0.9985	0.9983	*	0.0020	0.0087	0.0210	0.0072	0.0099	0.0107	0.0040	0.0630
4	0.9995	0.9995	0.9980	*	0.0050	0.0140	0.0030	0.0042	0.0040	0.0022	0.0692
5	0.9912	0.9920	0.9913	0.9950	*	0.0060	0.0069	0.0080	0.0050	0.0064	0.0460
6	0.9810	0.9810	0.9790	0.9863	0.9940	*	0.0136	0.0090	0.0062	0.0130	0.0545
7	0.9966	0.9960	0.9928	0.9970	0.9931	0.9870	*	0.0030	0.0020	0.0001	0.0641
8	0.9949	0.9950	0.9900	0.9960	0.9920	0.9910	0.9970	*	0.0008	0.0027	0.0800
9	0.9950	0.9950	0.9893	0.9960	0.9950	0.9920	0.9980	0.9992	*	0.0021	0.0690
10	0.9976	0.9973	0.9960	0.9978	0.9936	0.9870	0.9999	0.9970	0.9979	*	0.0649
11	0.9287	0.9280	0.9390	0.9331	0.9550	0.9470	0.9379	0.9228	0.9330	0.9372	*

midway between the African and European populations, probably as a result of introgressive hybridization between them. A gene flow of about 50% from the African population to the Aegean population was established.

Evidence for the gene flow from the northwestern part of the African coast (Mediterranean) to the Aegean Sea (in the remote past) is the frequency of the allele B in the first polymorphic zone, ( $q_{\text{PROT-1*B}}$ ). In the Cape Blank population, the frequency is 1 while in

the Aegean Sea it is 0.366-0.448. The frequency of this allele north of Canary Island is 0.046, and varies from 0.023 in the Black Sea to 0.160 near Valencia. This is a proof of the penetration of the European anchovy from the Atlantic Ocean along the European coast (northern part of Mediterranean and Black Sea). The analyzed populations from the Black, Marmora, Azov, Adriatic, and Mediterranean Seas (Nice and Valencia) are genetically close to the European anchovy caught to the north of Canary Island

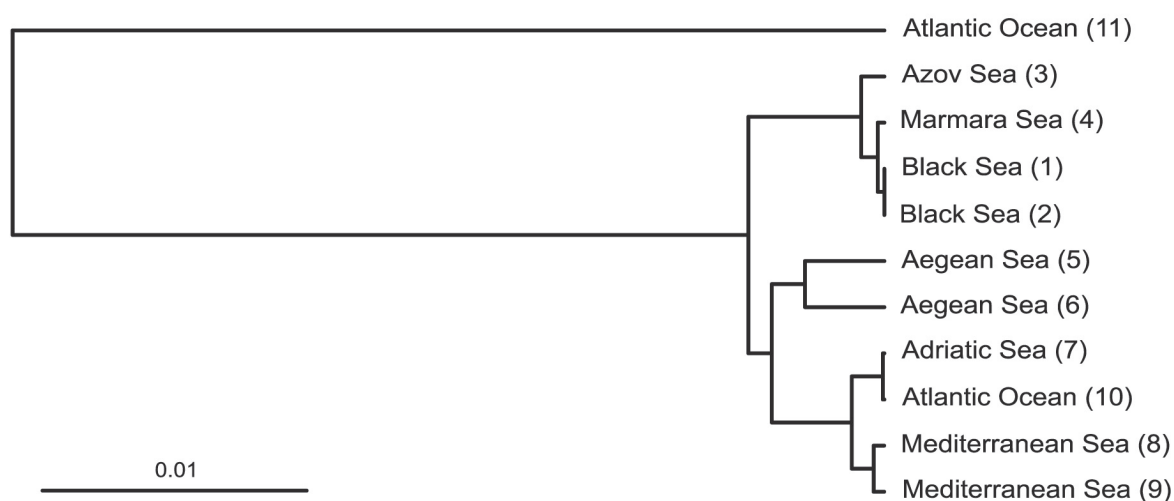


Fig. 4. UPGMA tree, derived from allozyme data for 22 loci using NEI'S genetic distance ( $D_{Nei}$ ), illustrating the relationships among 11 anchovy populations

No genetic-biochemical evidence for the existence of two anchovy subspecies (races), Atlantic and Mediterranean, has been found. Such evidence is found only for the European anchovy and this from the Cape Blank (African anchovy). Presumably the European anchovy populations are well diverged from the African one (Fig. 4).

DOBROVOLOV (1987, 1992) recognized that the Black and Azov anchovy populations had a common ancestor, the Atlantic anchovy that inhabits the European coast. A short genetic distance ( $D_{Nci}$ ) between the Black Sea and the Marmara Sea anchovy was also found.

In the late Miocene (about 6 million years ago), prolonged movements of the African continental plate northward broke the link between the Mediterranean and the Atlantic Ocean in the Gibraltar area (HSŮ, 1978). Communication with the Atlantic was closed and the Mediterranean was converted into a series of saline lakes. Very few marine species seem to have survived this salinity crisis that lasted several hundred thousands of years (HSŮ, K.J. 1978; POR & DIMENTMAN 1985; SELLI, 1985). The great bulk of present-day Mediterranean species entered the region during the early Pliocene, slightly less than 5 million years ago, when communication with the Atlantic was reestablished and Atlantic waters rushed through the Gibraltar into the Mediterranean (SARA, 1985; POR, 1989). It is supposed that the African anchovy occupied areas with warmer water along the Mediterranean coast of Africa, while the anchovy from the European coast of the Atlantic Ocean, being adapted to colder waters, settled along the European coast of the Mediterranean.

At the end of Pliocene, a connection was established between the Black and Aegean Seas through the Dardanelle and Bosphorus Straits (BACESCU, 1985, TORTONESE, 1985). The insignificant genetic distance between the Black Sea and the Atlantic anchovy, and between the Black Sea and the Adriatic populations, is evidence of the entry of the European anchovy into the Black Sea through its last connection

with the Mediterranean. The data for the genetic distances and the time of divergence be obtained had confirmed conclusions of SHEVCHENCO (1980).

The genetic divergence ( $D_{Nci}$ ) between the Azov and the Black Sea anchovy populations shows that they belong to two different populations, i.e., the Azov anchovy is not a Tercier relict as supposed by POUSANOV (1936). Probably some subspecies differences between them existed in the past but disappeared as a result of introgressive hybridization.

## CONCLUSIONS

No evidence for the existence of subspecies differentiation between anchovy populations from the European coast of the Mediterranean and the Atlantic Ocean was found. However, based on genetic-biochemical data, the existence of two anchovy subspecies, European and African, is suggested. The former inhabit the Atlantic and Mediterranean coasts of Europe and the Aegean, Marmora, Black, and Azov Seas. The latter inhabits the Cape Blank region in the Atlantic Ocean and, probably, the northwestern part of the African coast (Mediterranean).

The Aegean anchovy is a hybrid population as a result of introgressive hybridization between the European and the African anchovies. The Black, Marmora, Azov, Adriatic, and Mediterranean (Nice and Valencia) populations are genetically close to the European anchovy, caught north of the Canary Island. The genetic distance between the Azov and Black Sea populations show that the former probably entered the Black Sea during the Karangad period and the latter during the last connection of the Black Sea to the Mediterranean.

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**Genetske strukturne populacije brgljuna (*Engraulis encrasicolus*  
Linnaeus, 1758) (Osteichthyes: Engraulide)  
iz Mediterana i Atlantskog oceana**

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**SAŽETAK**

Analizirani su mišićni proteini europskog brgljuna (*Engraulis encrasicolus* L.) upotrebom škrobno gelne elektroforeze i izoelektričnog fokusiranja na tankom poliakrilamidnom gelu. Analizirana su 22 proteinska položaja i kod šestorice je dobiven polimorfizam. Postavljena je hipoteza na osnovi genetsko-biokemijskih podataka da postoje 2 podvrste brgljuna: europska i afrička. Prva naseljava Mediteran, mediteranske obale Europe, te Egejsko, Mramorno, Crno i Azovsko more. Druga je nađena u području Cape Blank u Atlantskom oceanu i vjerojatno uzduž sjeverozapadnog dijela afričke obale. Egejski brgljun se sastoji od hibridnih populacija koje su rezultat introgresivne hibridizacije. Nema uvida u diferencijaciju podvrsta između populacija s europske obale Mediterana i Atlantskog oceana. Genetske udaljenosti između populacija Azovskog i Crnog mora ukazuju da je prva ušla u Crno more tijekom karangadskog razdoblja, a druga tijekom zadnje veze Crnog mora i Mediterana. Genetska udaljenost između brgljuna iz Crnog mora i brgljuna iz Azovskog mora upućuje na to da se mogu razlikovati različite populacije. Vjerojatno su neke razlike u podvrstama nastale kao rezultat introgresivne hibridizacije.

**Ključne riječi:** brgljun, strukturna populacija, genetička udaljenost, Mediteranski bazen, Atlantski ocean

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