ISSN: 0001-5113 AADRAY	ACTA ADRIAT., 50(1): 77 - 90, 2009	UDC: 597.541:591.1(262.5+262.53+262.4) 597.541:591.15(262.5+262.53+262.4)

Morphologic and Allozyme Analyses of European anchovy (*Engraulis encrasicolus* (L. 1758)) in the Black, Marmara and Aegean Seas

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The morphologic and genetic variations of Engraulis encrasicolus *(L. 1758) were studied based on morphometric, meristic and allozyme analyses. Samples were collected throughout the Black, Marmara and Aegean Seas. Discriminant function analysis of both morphometric and meristic characters indicated the existence of four morphologically differentiated groups of* E. encrasicolus*. The Aegean Sea and Marmara Sea samples were the most isolated from all others for morphometric and meristic characters. Genetic analyses indicated great variability but suggested low levels of differentiation in the eastern Black Sea samples. However, the low genetic distance between this and other samples suggests the existence of hybridization between the Azov and eastern Black Sea populations. Nonetheless, genetic analysis showed that these levels were significant and the population structure should be analysed using markers able to detect a greater degree of population differentiation.*

Key words: population structure, anchovy, *Engraulis encrasicolus*, morphological variation, allozyme

INTRODUCTION

Marine pelagic fish tend to be distributed over extensive geographic areas where no clear geographic and oceanographic barriers are present. Stocks are distributed over a continuum of environmental conditions, as they migrate and mature at different sizes, spawn at more than one location and period, recruit in different periods and sizes, feed, grow and survive (BEGG *et al*., 1999). To manage a fishery effectively, it is important to understand the stock structure of a species and how fishing effort and mortality are distributed (GRIMES *et al*., 1987).

Various stock identification techniques have been employed to elucidate the temporal and spatial discreteness of fish stocks (IHSSEN *et al.,* 1981; MACLEAN & EVANS, 1981; NELSON *et al*., 1989; PAWSON & JENNINGS, 1996; AYVAZIAN *et al.,* 2004). Morphometrics and meristics are the two types of morphologic characters that have been most frequently used to delineate stocks of a variety of exploited fish species (MURTA, 2000; SILVA, 2003; O'REİLLY & HORN, 2004; TURAN, 2004; TURAN *et al.,* 2006). Allozyme electrophoresis has long been used to discriminate between fish stocks that are genetically isolated to varying extents (AYVAZIAN *et al.,* 2004; RYMAN & UTTER, 1987; AVISE, 1994).

The European anchovy, (*Engraulis encrasicolus* (L., 1758)) is a shoaling clupeoid fish, distributed along the eastern Atlantic coast from Scandinavia to western Africa, and also found in the Mediterranean, Black and Azov Seas (WHITEHEAD *et al*., 1988). However, recent evidence suggested that its distribution may extend as far south as Southern Africa, as well as in a portion of the Indian Ocean (GRANT & BOWEN, 1998; BORSA *et al*., 2004). As a consequence of its broad distribution and the existence of oceanographic barriers, the species may be composed of multiple disjunct populations. There have been a number of population structure analyses of *E. encrasicolus* carried out in Mediterranean and Atlantic waters which report morphometric and genetic differences between populations (SPANAKIS *et al*., 1989; BEMBO *et al*., 1996a, b; MAGOULAS *et al*., 1996; PLA *et al*., 1996; TUDELA, 1999; TUDELA *et al*., 1999; BOUCHENAK-KHELLADI *et al*., 2008; SANZ *et al*., 2008; KRISTOFFERSEN & MAGOULAS, 2008).

Although extensive biological and fisheries studies on anchovy have been carried out in Turkish waters (OZDAMAR *et al.,* 1994; DUZGUNES & KARACAM, 1989; MUTLU *et al.,* 1993; GOZLER & CILOGLU, 1998; CIHANGIR & USLU, 1992; CIHANGIR, 1994; KIDEYS *et al.,* 1999) there is limited information available on the population structure of anchovy in Turkish waters. The status of populations of anchovy in Turkish seas was preliminary investigated using morphometric characters by TURAN *et al.* (2004) which revealed a high degree of dissimilarity among the anchovy populations.

In Turkish waters, *E. encrasicolus* supports a large fishery in the Black Sea and comprises 63% of the total Turkish catch of this species and represents about 80% of the total fish production in the Black Sea (CIHANGIR & TIRAŞIN, 1991). The relative contribution of *E. encrasicolus* to local fisheries similarly follows for the Marmara Sea and Aegean Sea. In this context, the evaluation of the exchange of individuals along the Turkish coast is important for fisheries management.

The aims of this study are to (1) examine the population genetic structure of anchovy using allozyme electrophoresis from throughout its range, focusing on the Black Sea, and (2) to compare the population structure based on morphometric characters using the "Truss network system" and meristic characters from Turkish seas.

MATERIAL AND METHODS

Sample

A total of 300 anchovy specimens were collected by commercial fishing vessels from six fishing areas, three from the Black Sea (Trabzon, Sinop, Istanbul), one from the Marmara Sea (Bandırma Gulf) and two from the Aegean Sea (Edremit Gulf, Izmir Gulf) between November 2001 and January 2002 (Table 1; Fig. 1). Following the capture, samples were placed individually into plastic bags and were kept deep-frozen (-20 °C) until transportation to the laboratory. Samples of white muscle were removed from individuals and stored at –80 °C until further treatment for allozyme analysis.

Morphometric and meristics

Morphometric and meristic data were collected from all samples. Sex was determined

Table 1. Sampling details of E. encrasicolus *used in this study*

Sampling area	Abbreviation	Sample size	Sex (M/F)	Mean STL	Range of STL
Eastern Black Sea (Trabzon)	BS1	50	28/22	10.48±0.07	9.45-11.7
Central Black Sea (Sinop)	BS2	50	16/34	10.04±0.09	8.75-11.35
Western Black Sea (Istanbul)	BS3	50	11/39	10.28±0.06	9.35-11.05
Marmara Sea (Bandırma)	MS	50	43/7	11.34±0.06	10.5-12.1
Northern Aegean Sea (Edremit)	AS1	50	16/34	10.34±0.05	9.5-11.2
Aegean Sea (İzmir)	AS2	50	18/32	10.14±0.06	9.15-11.45

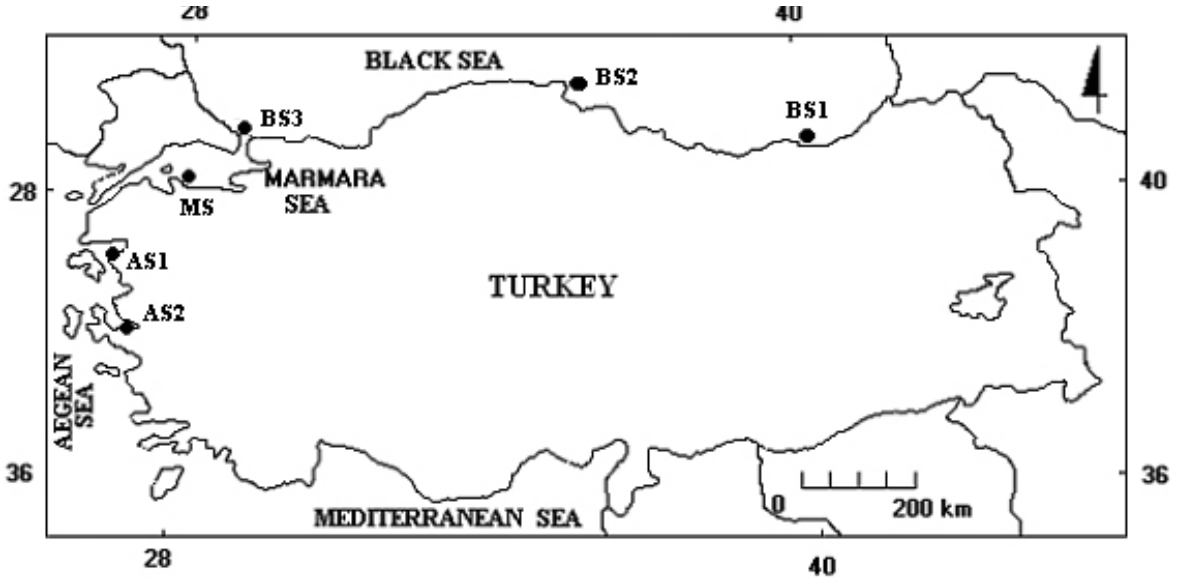

Fig. 1. Sampling locations of anchovy. Abbreviations of the locations are given in Table 1

macroscopically whenever possible (Table 1). Morphometric data were collected using the "Truss network system". Data points were arranged in "trusses" around the fish (Fig. 2), a layout which maximises the number of measurements and increases the sensitivity of the analysis (STRAUSS & BOOKSTEIN, 1982). Fish were laid out on a piece of polystyrene board and fixed into position by the insertion of pins along the body. This enabled accurate and consistent measurements. Each landmark was obtained by piercing the acetate sheet with a dissecting needle, defining 12 landmarks. Additional data, such as eye diameter (ED), head width (HW), pectoral fin length (PL) and pectoral fin width (PW) were also recorded. Measurements were made to the nearest 0.01 mm using calipers. Meristic counts were made of six meristic body characters: pectoral (P), anal (A), ventral (V) and dorsal (D) fin rays and upper (UGR) and lower (LGR) gill rakers. All the meristic counts were made under a binocular microscope.

Separate statistical analyses were conducted on the morphometric and meristic data. Character differences among populations were analysed using univariate and multivariate statistics. One-way analysis of variance (ANOVA) was performed for the comparison of the morphometric differences between the two sexes. Co-variation of characters was investigated by multivariate analysis of variance (MANOVA). Most of the variability in a set of multivariate characters is due

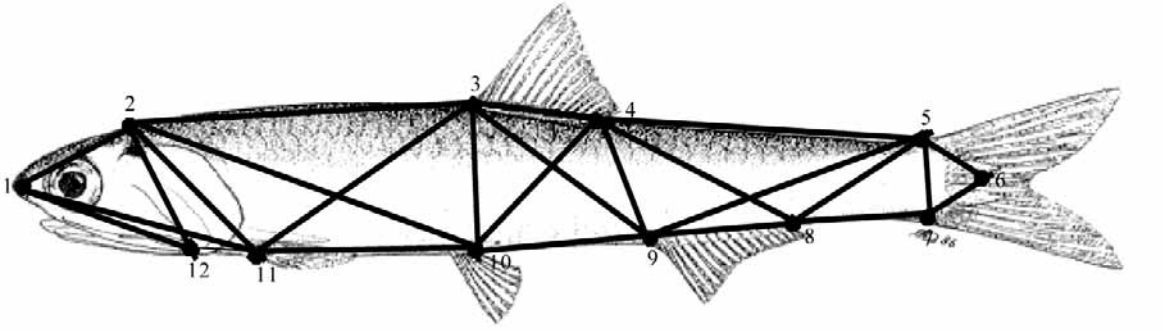

Fig. 2. Locations of the 12 landmarks for constructing the truss network on E. encrasicolus *illustrated as black dots and morphometric distance measures between the dots as lines*

to size (JUNGUERA & PEREZ-GANDARAS, 1993). Thus, shape analysis should be free from the effect of size to avoid misinterpretation of the results (STRAUS, 1985). No significant correlations were observed between meristic characteristics and standard length of samples. However, significant correlations were observed between size and morphometric characteristics between the samples. Therefore, transformation of absolute measurements to size-independent shape variables was the first step of the analyses. In order to eliminate any variation resulting from allometric growth, all morphometric measurements were standardised according to ELLIOTT *et al*. (1995).

$$M_{adj} = M\,(L_s/L_o)^{\,b}$$

where M is the original morphometric measurement, M_{adj} is the size-adjusted measurement, L_o is the standard length of fish, and L_s is the overall mean of standard length for all fish from all samples for each variable.

The parameter b was estimated for each character from the observed data as the slope of the regression of log M against log L_o, using all specimens.

Correlation coefficients between transformed variables and standard length were calculated to check if the data transformation was effective in removing the effect of size from the data. The standardised truss measurements showed no significant correlation with standard length. Therefore, the size effect had been successfully removed with the allometric transformation. Discriminant function analysis (DFA) was used to determine the dissimilarity between populations. The statistical packages SPSS and Statistica for Windows were used for the statistical analyses.

Allozyme

The samples (BS1, Trabzon; BS3 Istanbul; MS, Bandırma; AS2, Izmir) that showed morphologic differentiation were chosen for genetic analysis. Allozyme analysis was carried out employing standard horizontal starch-gel electrophoresis (MORITZ & HILLS, 1990). Nomenclature for enzyme loci and allele designation follows according to SHAKLEE *et al.* (1990). After an enzyme screening program, two enzymes comprising two putative loci that produced well-resolved staining patterns consistent with known enzyme sub-unit structures were routinely examined. The enzymes used were: glycerol-3-phosphate dehydrogenase (*G3PDH**, E.C. 1.1.1.8) and phosphoglucose mutase (*PGM**, E.C. 5.4.2.2). Alleles were scored according to their mobility relative to the most commonly observed allele which was designated as *100. A locus is considered polymorphic if the frequency of the most common allele does not exceed 0·99 ($P_{0.99}$) or 0.95 ($P_{0.95}$). Nei's genetic distances were used to estimate genetic relationships between species using the neighbour-joining (SAITOU & NEI, 1987) method. A dendrogram to illustrate the genetic divergence among the examined populations was constructed from genetic distances using the unweighted pair-group method using arithmetic means (UPGMA) (SNEATH & SOKAL, 1973). Robustness of nodes in the neighbour-joining tree was evaluated by bootstrapping over samples (FELSENSTEIN, 1985). All calculations were performed using TFPGA*v*1.3 (MILLER, 1997) and BIOSYS Release 1.7 (SWOFFORD & SELANDER, 1989).

RESULTS

Morphometric

Univariate statistics (ANOVA) showed no statistical differences between males and females for morphometric and meristic variables ($P>0.05$), so sexes were pooled in further analysis.

In discriminant function analysis, the first canonical function accounted for the largest amount of between–group variability (46%) while the second and third accounted for 23% and 12% respectively. Plotting DF1 and DF2 explained 69% of the between-group variation and revealed clear between-population differences (Fig. 3). The Aegean Sea samples (AS1 &

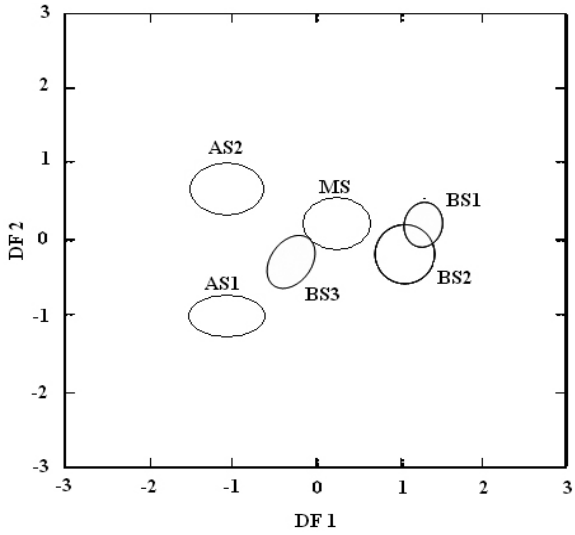

Fig 3. Discriminant function analysis plot with 95% confidence ellipses for morphometric analysis

AS2) were the most isolated from each other and from all other samples. The western Black Sea sample (BS3) was also clearly separated from the other Black Sea samples, but was closer to the Marmara Sea sample (MS). The middle (BS2) and eastern Black (BS1) sea samples were overlapping together.

The important discriminative characters in distinguishing between the groups for the first and second discriminant functions were from the body height measurements (3-9) (Table 2). Using these morphometric characters each specimen could be classified correctly to the original populations with an accuracy of 77% (Table 3). The proportion of those correctly classified into their original group was highest (100%) for the Aegean Sea sample (AS2).

Table 2. Results of discriminant function analysis (DFA). (variables ordered by activity degrees in distinguishing of populations)

	Function						
Characters	DF1	DF2	DF3	DF4	DF5	DF6	DF7
3-9	0.870	-0.110	-0.022	0.072	0.004	0.009	-0.037
1-11	0.866	0.121	0.237	-0.086	0.140	0.018	0.003
4-8	0.856	-0.146	0.009	0.062	0.013	-0.002	0.034
2-10	0.850	0.063	0.066	0.231	0.034	-0.063	-0.033
9-10	0.804	-0.100	-0.122	0.062	-0.066	0.081	0.023
4-9	0.803	-0.245	-0.126	0.116	-0.052	-0.016	0.031
1-12	0.800	0.060	0.240	-0.193	-0.099	0.037	-0.032
3-11	0.764	-0.070	-0.090	0.281	-0.168	-0.042	-0.045
2-3	0.756	0.066	-0.051	0.191	-0.062	0.022	0.048
4-10	0.743	-0.123	-0.025	0.128	-0.204	0.145	-0.006
5-9	0.634	0.199	-0.239	-0.131	0.117	0.022	0.006
4-5	0.632	0.215	-0.299	-0.073	0.190	-0.001	0.020
HW	0.631	-0.570	0.006	-0.264	0.025	-0.024	-0.029
2-11	0.618	0.113	0.376	0.064	0.070	0.123	0.118
10-11	0.600	0.010	0.023	0.308	-0.068	-0.162	0.184
5-8	0.593	0.454	-0.504	-0.163	0.128	-0.002	-0.020
1-2	0.585	0.192	0.322	-0.393	-0.063	-0.113	-0.081
ED	0.549	0.319	0.284	-0.097	-0.192	-0.193	-0.026
2-12	0.517	0.388	0.420	-0.263	-0.148	0.020	-0.023
7-8	0.515	0.502	-0.501	-0.204	0.086	-0.008	-0.050
PW	-0.296	0.838	0.111	0.238	-0.065	0.037	0.008
PL	0.645	-0.653	-0.091	-0.140	0.037	-0.026	-0.006
3-10	0.208	0.125	-0.088	0.471	-0.379	0.167	0.176
11-12	0.341	0.127	0.143	0.174	0.591	0.002	0.097
3-4	0.0455	-0.022	0.134	0.035	0.210	0.857	-0.316
5-7	0.0650	0.0262	-0.100	-0.292	-0.230	0.332	0.755
8-9	0.0139	-0.06364	0.218	0.248	0.441	-0.152	0.484

Table 3. Correct classification showing the percentage of specimens classified in each group

Samples	Group					
	BS1	BS2	BS3	MS	AS1	AS2
BS1	**60**	6	12	16	6	0
BS2	20	**62**	6	6	6	0
BS3	6	12	**74**	2	6	0
MS	8	4	4	**84**	0	0
AS1	0	8	6	4	**82**	0
AS2	0	0	0	0	0	**100**

Table 4. Results of discriminant function analysis (DFA). (variables ordered by activity degrees in distinguishing of populations)

	Function		
Characters	DF1	DF2	DF3
UGR	0.871	0.131	0.05643
LGR	0.857	0.158	0.137
P	0.141	0.656	0.423
V	0.319	0.605	0.280
A	0.08684	0.513	0.407
D	0.02314	0.353	0.757

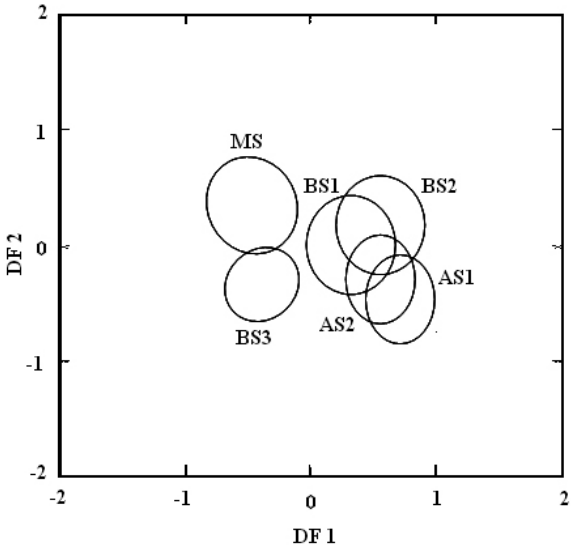

Fig. 4. Discriminant function analysis plot with 95% confidence ellipses for meristic analyses

Meristics

Plotting DF1 and DF2 accounted for 76% of total variance and showed a clear inter-sample differentiation (Fig 4). The Marmara Sea sample (MS) overlapped with the western Black Sea sample (BS3) and was separated from all other samples.

The most important discriminative meristic characters in distinguishing between the groups for the first and second discriminant functions were upper (UGR) and lower (LGR) gill rakers (Table 4) Using all meristic characters, each specimen could be classified correctly to the original populations with an accuracy of 36.3% (Table 3). Meristic characters showed lower differentiation in comparison to morphometric characters. The proportion of those correctly classified into their original group was the highest (50%) for the Marmara Sea samples (MS).

Table 5. Correct classification showing the percentage of specimens classified in each group

Samples	Group					
	BS1	BS2	BS3	MS	AS1	AS2
BS1	**32**	6	12	20	18	12
BS2	14	**26**	12	14	18	16
BS3	14	0	**36**	24	10	16
MS	10	6	14	**50**	6	14
AS1	18	4	8	10	**32**	28
AS2	16	8	8	10	16	**42**

Table 6. Allele frequencies at polymorphic loci and genetic diversity parameters in E. encrasicolus

			Mean heterozygosity	
Population	Mean sample size per locus	Mean no. of alleles per locus	Direct count (H_o)	Expected (H_E)
BS1	15 (0.0)	2 (0.0)	0.067 (0.000)	0.329 (0.041)
BS3	15 (0.0)	1 (0.0)	0.000 (0.000)	0.000 (0.000)
MS	15 (0.0)	1 (0.0)	0.000 (0.000)	0.000 (0.000)
AS2	15 (0.0)	1 (0.0)	0.000 (0.000)	0.000 (0.000)

Table 7. Frequencis of alleles found fo each locus among the samples of E. encrasicolus

Locus	Alleles	BS1	BS3	MS	AS2
n	15	15	15	115	15
G3PDH	100	0.767	1.000	1.000	1.000
	156	0.233	0.000	0.000	0.000
PGM	100	0.833	1.000	1.000	1.000
	50	0.167	0.000	0.000	0.000

Allozyme

Two loci, *G3PDH* and *PGM*, were polymorphic in eastern Black Sea (BS1) samples. The mean number of alleles per locus was 2. The values of observed mean heterozygosity (H_o) was 0.067, and the expected mean heterozygosity (H_e) was 0.329 (Table 5). These two loci significantly deviated from the Hardy-Weinberg equilibrium ($P<0.001$). Allelic frequencies for the two scored polymorphic loci are listed in Table 6.

NEI's (1978) genetic distance (D) between the eastern Black Sea (BS1) and the other samples was found to be 0.024 (Table 7). The eastern

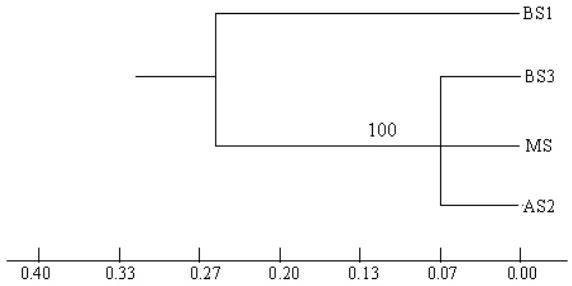

Fig. 5. UPGMA dendrogram showing genetic distance between anchovy samples, based on Nei, 1978. Bootstrap value is given on the node

Black Sea population (BS1) appeared to be in genetic differentiation. This differentiation was also clearly illustrated in the dendrogram derived from UPGMA cluster analysis (Fig 5).

DISCUSSION

The results obtained from body morphometrics and meristics in this work indicate the existence of morphologically differentiated groups of *E. encrasicolus* in Turkish territorial waters. On the other hand, genetic analysis showed indications of differentiation of the eastern Blacks Sea samples from Trabzon (BS3).

Marmara Sea (MS) samples exhibited a marked separation from all others for both meristic and biological characters. On the other hand, there is notable intermingling between the Marmara and neighbouring population from the Black Sea (BS3). The Marmara Sea is the passageway between the Black Sea and Aegean Sea, and currents or water masses play an important role in its environmental conditions (e.g. temperature, salinity, food). Most authors agree that environmental conditions play the largest part in determining morphological variation (WINANS, 1984). Hence the variation observed in Marmara Sea samples (MS) may be attributable to the productivity and temperature differences within this sea, presumably representing growth and development in contrasting waters. Many papers have reported that the final number of structures achieved by meristic attribute is determined by the environmental characteristics prevailing during a critical stage in the development of the individuals, during which they are more phenotypically influenced by the environment (TUDELA, 1999). On the other hand, the detected pattern of phenotypic discreteness between the samples may suggest that the phenetic relationship between the populations increases with geographical distance. In Turkish coastal waters, Black Sea anchovy migrates into the Marmara Sea in autumn to overwinter and migrates back into the Black Sea for feeding and spawning in spring (DEMIR, 1974; DANILEVSKY, 1961). It is possible that the Marmara Sea anchovy behave similarly in that they migrate during summer into the Black Sea and spawn along the Turkish coast (GORDINA *et al.,* 1997).

The detected genetic differentiation in two loci for the eastern Black Sea population (BS1) may indicate that there may be a genetically different population in Turkish territorial waters. According to some researchers, Azov anchovy migrates through the Kerch Strait between the Azov Sea and the Black Sea for feeding and may form a hybrid with the Black Sea anchovy (GORDINA *et al.,* 1997; CHASHCHIN, 1985). At times of unfavourable food conditions the Black Sea anchovy migrates to the Azov Sea to feed, and in some years it also spawns there (GORDINA *et al.,* 1997; DANILEVSKY, 1960). In a number of preced-

Table 8. Nei's (1978) genetic distance (D) between four populations of anchovy based on allozyme analyses

Population	BS1	BS3	MS	AS2
BS1	—			
BS3	0.024	—		
MS	0.024	0.000	—	
AS2	0.024	0.000	0.000	—

ing papers (DANILEVSKY, 1960; ALTUKHOV, 1974; MARTY, 1980) an overlapping of the ranges of Azov and Black Sea anchovy was noted. In winter individuals of both races are frequently fished in the same areas. Even in summer they can both be caught in the Sea of Azov, where juveniles of Black Sea anchovy enter to feed. They also coinhabit less saline waters in the northwest Black Sea accessible to Azov anchovy (CHASCHIN, 1996). Several researchers have reported population differences within and between the Black Sea and Azov anchovy. ALTUKHOV *et al.* (1969) found differences based on immunological analyses between the Black Sea and Azov anchovy. KALNIN & KALNINA (1984, 1985) found significant differences between the Azov and Black Sea and suggested that there are two distinct populations of anchovy in the Black Sea. IVANOVA & DOBROVOLOV (2006) found genetic divergence between the Azov and the Black Sea anchovy populations and suggest that Azov and Black Sea anchovy belong to different populations.

In our study, NEI's (1978) genetic distance (D) between the eastern Black Sea (BS1) and the other samples was found to be 0.024. TUDELA *et al.* (1999), found genetic homogeneity in anchovy reproducing between southern Catalonia and Tuscan archipelago (genetic distance D less than 0.001), and concluded that they belonged to a single genetic population. This conclusion leads us to suggest an environmental basis for the morphologic differences described in the present study. A high degree of morphologic differences lacking geographical basis and independent of genetic population structure (SPANAKIS *et al.,* 1989; TUDELA, 1999) has also been reported in *Engraulis mordax.*

In the present study, the observed mean heterozygosity (Ho) was 0.067 and that was similar to values for *E. japonicus*. Genotypic proportions deviated significantly from the Hardy-Weinberg equilibrium ($P<0.001$) in the eastern Black Sea (BS1) samples. This may be the result of selective forces against heterozygotes in the system (ARCULEO *et al.,* 2003).

In summary, the observed morphometric and meristic differentiation between the stocks indicate that there is stock structuring of anchovy in Turkish territorial waters that is in agreement with the previous morphometric study by TURAN *et al.* (2004) who reported morphometric differences between the Black and Aegean Seas. However, the genetic basis of this differentiation was not revealed here. This is most probably due to an insufficient number of loci and genetic techniques used in this study. Although the environmental factors may be governing to some degree the potential phenotypic differentiation of *E. encrasicolus* populations, the detected pattern of genetic variation in the eastern Black Sea samples (BS1) suggests that there may be a self-recruiting population or sub-species of anchovy in the Black Sea. Given that existing genetic differentiation seems to be weak, it becomes of great importance to use molecular markers with higher polymorphism, such as microsatellites, which have been able to detect a greater degree of population differentiation than allozymes (SHAW *et al.,* 1999). Also, in future studies, analysing a higher number of polymorphic enzymes would increase genetic heterogeneity among the anchovy populations, which may support the detected phenotypic differentiation.

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Received: 12 January 2009

Accepted: 3 April 2009

Morfološka i analiza alozima europskog inćuna (*Engraulis encrasicolus* (L. 1758)) u Crnom, Mramornom i Egejskom moru

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SAŽETAK

Morfološke i genetske varijacije inćuna, *Engraulis encrasicolus* (L. 1758), su proučavane na osnovu alozima, te morfometričkih i merističkih karakteristika. Uzorci su prikupljeni u Crnom, Mramornom i Egejskom moru. Analiza morfometričkih i merističkih karakteristika je ukazala na postojanje četiri morfološki različite grupe inćuna (*E. Encrasicolus*). Primjerci iz Mramornog i Egejskog mora su se značanjno razlikovali od drugih primjeraka zbog morfometričkih i merističkih karakteristika. Genetska analiza ukazuje na veliku varijabilnost, ali i na nizak nivo diferencijacije kod uzoraka iz Crnog mora. Niska genetska udaljenost između ovih i drugih primjeraka ukazuje na postojanje hibridizacije između Azovske i populacije istočnog Crnog mora. Usprkos tome što je genetska analiza pokazala da su ovi niovi značajni, struktura populacije bi se trebala analizirati pomoću markera koji bi otkrili veći stupanj raznolikosti populacije.

Ključne riječi: struktura populacije, inćun, *Engraulis encrasicolus*, morfološke varijacije, alozimi