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RESEARCH PAPER

Genetic Diversity for Anchovy Populations (*Engraulis encrasicolus L.)* in the Azov, Marmara and Black Sea

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Abstract

In this study, the structure of the anchovy population in the Azov, Marmara and Black Sea was determined by restriction fragment length polymorphism (RFLP). Sampling was carried out at six stations represented the Azov, Marmara and Black Sea populations in 2011. Total DNA of the sample was taken each station and the mtDNA D-loop region within total DNA was amplified by PCR. Amplified mtDNA D-loop region by PCR (polymerase chain reaction) was cut by10 different enzymes (*Hpa II, Hinf I, Hae III, Alu I, Ava II, Nci I, RsaI, TaqI, Tru91, Hsp9211)* and morphs were determined by the type of the particle profiles for each population. mtDNA D-loop haplotypes were determined by combining this morphs. As a result, a total of 64 haplotypes belong to populations that were sampled from six stations (Azov Sea, Black Sea; Georgia, Abkhazia, Trabzon-Turkey winter, Trabzon-Turkey summer and Marmara Sea) have been identified. Fst values among populations were calculated by the analysis of molecular variance and the Marmara Sea populations clearly separated from other populations. Also it was seen that Trabzon-summer samplings were sharing haplotypes of all other populations. No evidence have found for recent population bottleneck of the population studies.

Keywords: Anchovy, *Engraulis encrasicolus,* Population genetics, RFLP, mtDNA.

Introduction

The anchovy is the one of the common pelagic fishes with million tons of catch rate per year all around the World. The European anchovy distributed from the eastern Atlantic coast to West Africa and Scandinavia and in the Mediterranean, Black and Azov Seas (Whitehead, Nelson, & Wongratana, 1988). However, recent studies have suggested that its range may even extend from a part of the Indian Ocean to Southern Africa (Grant & Bowen, 1998; Borsa, 2004). Morphological characteristics of the anchovy depicting geographical differences indicate that several subspecies or races can be seen in the Mediterranean. (Spanakis, Tsimenides, & Zouros, 1989). Several researchers reported that several significant populations may be found within Mediterranean basin and they suggested that two subspecies of anchovy lived in both the Black Sea and Azov Sea (Alexandrov, 1927; Chashchin, 1996; Spanakis *et al.*,1989; Bembo, Carvalho, Cingolani, Arneri, Giannetti, & Pitcher, 1996a; Bembo, Carvalho, Cingolani, & Pitcher, 1996b; Magoulas, Tsimenides, & Zouros, 1996; Magoulas, Castilho, Caetano, Marcato, & Patarnello, 2006; Borsa, 2002).

Alexandrov (1927) informed that the Black Sea anchovy lives in the western part of the Black Sea while the Azov anchovy lives in the eastern section of the Black Sea and they came in to the Azov Sea as large groups for reproduction and nurture. Kalnin and Kalnina (1984,1985) and Kalnina, Kalnin, & Dashova (1984) investigated population model, gene flow, biochemical polymorphism, genetic variation, and reproductive isolation of two anchovy subspecies in the Black and Azov Seas. Later studies have described genetic divergence between the Azov and the Black Sea anchovy populations and suggest that Azov anchovy (*Engraulis encrasicolus maeticus*) and Black Sea anchovy (*Engraulis encrasicolus ponticus*) belong to different populations (Dobrovolov, 1987, 1992; Ivanova & Dobrovolov, 2006). Erdoğan, Turan, & Koç (2009) studied allozyme and morphologic analyses and found out genetic variations in anchovy (*E. encrasicolus)* population of the Black, Marmara and Aegean Seas.

There are several studies carried out throughout the years related to growing (Levi, Andreoli, & Arneri, 1994), morphology, morphometric, allozyme, nuclear DNA, or mtDNA variation of the European anchovy populations (Spanakis *et al.*, 1989; Magoulas *et al.*,

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1996; Bembo *et al*., 1996a, b; Borsa, 2002; Borsa, 2004). Cronin, Spearman, Wilmot, Patton, & Bickham (1993), Margoulas & Zouros (1993) used mtDNA markers for identification and detection intraspecific change of species in *E. encrasicolus* stocks and they reported two major anchovy mtDNA phylads for the Mediterranean. It was determined that phylad A was the dominant kind in the Black Sea and Aegean Sea while phylad B had higher number in western regions. (Margoulas & Zouros, 1993; Margoulas, *et al*., 1996). In addition, several studies have been carried on mitochondrial DNA to determine the stock structure.

Despite a few studies have been done related to European anchovy's genetic structure considering sustainable fisheries plans for the anchovy stocks, using RFLP method the comparative studies for genetic structure of the Marmara, Azov and Black Sea anchovy populations are not found. In the view of such information, we studied mitochondrial DNA polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay in order to determine the phylogenetic relationships among six anchovy populations in the Black Sea (South-east part), Azov Sea and Marmara Sea. Genetic differences among population of anchovy stayed on our Seas especially during summer period will be found out with this study.

Materials and Methods

Sample Collection and Storage

The anchovy samples were collected from the Azov, Marmara and Black Sea which are Abhazia, Georgia, Trabzon-Turkey in the winter period of 2011 year. In addition, it was collected only in Black Sea, Trabzon-Turkey in summer period of 2011. (Figure 1)

In this study pieces of tissue were taken from the caudal fin of the fish were put in containers with 98% ethanol and stored at -20°C in a deep freeze until DNA extraction. Eventually, samples were appropriately labelled and stored at -80°C for further studies.

DNA Extraction

A commercial kit was used for the purification of total DNA (Qiagen DNA mini kit). Quantity and quality of DNA was evaluated by means of spectroscopy (Bio-Rad SmartSpec Plus) and agarose gel 1,5 % in 1X TBE buffer (composed of: Trizma base 89 mM (*Sigma*), Boric acid 89 mM (*Sigma*) and EDTA 2 mM pH = 8 (*Sigma*)).

PCR Amplification

MtDNA Dloop region from the total DNA obtained from anchovy samples were amplified via a Thermal Cycler using primer sets (forward and reverse) developed by Cronin et aI.,1993. The following primer F5'-CTG AAA CTG CCC TAG TAG C -3' and R5'-GAC TAG CAC ACA AAC GAA AC -3' were used to amplify d-loop region. Amplification reactions were carried out with 50µl final volume and contained 2 µl forward and 2 µl reverse primers (10pmol), 25 µl 2X PCR Master Mix, (QIAGEN) and 19 µl ddH_2O and 2 µl (100 ng/µl) DNA

The reaction was programed on a Thermal cycler (Bio-Rad DNA-ENGINE, PTC 200) with an initial denaturation for 1 minutes at 95°C followed by 30 cycle at 95°C for 1 minute (denaturation) at 50°C 1 minute (annealing) at 70 °C 2 minutes (extension) and final extension at 70°C 10 minutes for D-loop. As a

Figure 1. Map of sampling location 1,fishing Season; 2, fishing Season and summer period (Trabzon-Turkey in May)

result of PCR rise, 4 µl of product was run in 1xTBE buffer system and 1% of agarose gel, stained with ethidium bromide, was viewed and checked with UV illuminator. Size distribution by molecular weight was determined with λDNA/*Hind*IIImarker (QIAGEN). The successful PCR products after control were stored at -20°C

Restriction Enzyme Analysis

Amplified mtDNA D-loop region by PCR restricted with use of restriction enzymes (*Hpa II, Hinf I, Hae III, Alu I, Ava II, Nci I RsaI, TaqI, Tru91, Hsp9211)* that were used in previous studies. D-loop PCR product is composed of the restriction enzyme and each restriction enzyme mixture consist of 7,45 µl sterile distilled water 1,4 µl 10X restriction enzyme buffer, 0,15 µl restriction enzyme (10µm) and 5µL PCR product. After the incubation of PCR product by restriction enzyme, the restricted samples were examined on 1,5% agarose gel with ethidium bromide (1mg 100 µl) in a 1x TBE buffer system. The gels were run for 1-2 h for the separation of reticted DNA fragments. Restricting Dloop PCR product was used to generate species specific restriction pattern. Estimating the genetic distances between haplotypes was accomblished by digestion profiles which produce a matrix of presence/absence of restriction sites. The program Total Lab version 2003 was used calculate the molecular size of restriction fragments comparing with 100bp ladder molecular weight marker

We used ARLEQUIN version 5 (Schneıder, Roessli, & Excoffier, 2000) to determine F_{ST} pairwise values and genetic heterogeneity in our study. Molecular variance analysis (AMOVA; Excoffıer, Smouse, & Quattro, 1992) was used to examine hierarchical population structure like population subdivision based on geographical strands. Also, GENALEX 6 (Peakall & Smouse, 2006) programme was used to determine the relationship between genetic difference and geographical distance.

Results

In total, 191 individuals were genotyped in this study. Two different restriction profiles were found for *Hpa II, Hinf I, Hsp9211* and *Ava II(A-B),* three for *RsaI, Hae III* and *Nci I (A-C),* six for *Tru91(A-F),* seven for *Taq(A-G) I,* one for *Alu I(A)* in 191 fish for which the composite 10 enzyme pattern was obtained. As a result of the study, total of 64 haplotypes were found in six populations. Haplotype and clade frequencies of all the samples were shown in Table 1

Genetic differences between samples were evaluated with method of Wright (1965) considering FST values which is indicator of genetic differences. Comparing of the samples were carried out with 1000 times permutation technique. Values of FST and significance were calculated with Arlequin software (Schneider *et al*., 2000) and shown in Table 2. According to results, it was understood that population of the Marmara Sea has distinctive character in contrast to other populations. The Azov anchovy population was similar to the Georgia and Abkhazia populations with 0.00858 and 0.14650 F_{ST} values while showing a distinctive character in contrast to the Marmara Sea anchovy population with F_{ST} value of 0.47754. It was seen that Trabzon-summer samplings were sharing haplotypes of all other populations. The Black Sea anchovy population was sampled in 2011 fishing season (winter) was different in contrast to the Marmara Sea, Abkhazia and Azov populations while similar to the Trabzon-summer population with 4 haplotypes and Georgia population with 3 haplotypes (Table 1). Relationships of geographical and genetic distance were found significant (r=0.96). Fst values of distant populations were more significant than F_{ST} values of closer populations while F_{ST} values of closer populations were insignificant (P<0.001).

Haplotype richness (R_H) and genotypic richness (R) of within populations is given in Table 3. The highest haplotypic richness (R_h) and genotypic richness (R) were determined as 15.928 and 0.684 respectively in the Trabzon summer population while the lowest values were determined in the Marmara population. On the other hand, Tajima's D test of selective neutrality was non-significant (P<0.001). (Table 3)

F_{ST} values among populations were calculated by molecular variance analysis (Table4). mtDNA variation determined by AMOVA analysis was occured at a rate of 23.03% among the populations and 76.97% in the populations. It was specified that variation components were significant statistically for both two comparisons (P<0.001) (Table 4).

Besides, clustered anchovy populations by the phylogenetic lineages that it contains applying principal coordinates analysis (PCoA) by Genalex 6 software. The first three axis are indicate 100% of total genetic variation. The highest variation values and Eigen values were found in 2. axis (Table 5). In the view of the analysis results, it is clear fact that the Marmara Sea anchovy population clustered different from other populations (Figure 2).

Discussion

It seems that the European anchovy has a significant degree of subdivided genetic population and phylogeographic complexity in comparison with other coastal pelagic fishes. The coastal pelagic species have a FST value of 0.01 or less. According to the results, the European anchovy mostly has an FST value of about 0.15. (Magoulas, 2006) In our study most high FST values was found as 0.47754 while the most low found as 0.00858 among 6 populations. Reasons for these dissimilarities, the European anchovy that distributed the Black Sea as a migratory fish is more complex genetic structure as well as having more sub-

Table 1. Distribution of MtDNA d-loop haplotype among anchovy population

HAPLOTYPES	NB	TOTAL	ABK*	AZ*	GEO*	TWIN*	MAR*	TSUM*
AAAAAAAAAA	1	23	7	10	6			
AAAAAAAAAB	2	8	2	4	2			
AAAAAAAAAC	3	2	2					
AAAAAAAAAD	4	1		1				
AAAAAAAAAE	5	1		1				
AAAAAAABAA	6	7		4	3			
AAAAAAABAB	7	14	2	5	7			
AAAAAAABAC	8	4	2	2				
AAAAAAACAA	9	2	1	1				
AAAAAAADAA	10	4	4					
AAAAAAADAB	11	6	5					1
AAAAAAAEAA	12	3	3					
AAAAAAAEAB	13	5	5					
AAAAAAAEAC	14	1	1					
AAAAAAAEBB	15	1	1					
AAAAAAAFAA	16	1						1
AAAAABAAAA	17	1		1				
AAAAACAAAA	18	12			4	5		3
AAAAACAAAB	19	4			2	2		
AAAAACABAA	20	6			1	4		1
AAAAACABAB	21	2				1		1
AAAAACABAC	22	1			1			
AAAAACABAF	23	1				1		
AAAAACADAA	24	16			1		13	2
AAAAACADAB	25	5					5	
AAAAACAEAA	26	5					5	
AAAAACAEAB	27	1					1	
AAAAACAEAC	28	3					3	
AAAAACAEAG	29	1					1	
AAAAACBBAA	30	1				1		
AAABAAADAB	31	1						1
AAABACADAA	32	1						1
AAACAAAAAB	33	2						2
AAACAAABAB	34	1						1
AAACAAABAC	35	1			1			
AAACAAADAA	36	4						4
AAACAAAEAA	37	2						2
AAACAAAEAB	38	1						1
AAACAAAEAC	39	2						2
AAACACAAAA	40	2						2
AAACACAAAB	41	1						1
AAACACABAA	42	1				1		
AAACACABAB	43	2				1		1
AAACACABAG	44	1						1
AAACACADAA	45	1						1
AAACACAEAG	46	1						1
AAACACAFAA	47	2						2
AABAAAAAAA	48	1		1				
AACCAAABAA	49	1						1
ABACACAAAA	50	1						1
BAAAAAAAAA	51	1	1					
BAAAAAADAA	52	2	2					
BAAAAAAEAA	53	1	1					
BAAAACAAAB	54	1				1		
BAABAAAAAA	55	3		1	2			
BAABAAABAA	56	1		1				
BAABACAAAA	57	2				2		
BAABACABAA	58	2				2		
BAABACABAB	59	1			1			
BAABACADAA	60	1					1	
BAABACADAB	61	2						2
BAABACADAG	62	1					1	
BAACAAADAB	63	1						1
BABBAAAAAA	64	1						1
	TOTAL	**191**	**39**	**32**	**31**	**21**	**30**	**38**

AZ:Azov Sea; GEO:Georgia; TWIN:Trabzon winter ; TSUM:Trabzon summer; MAR:Marmara Sea; ABK: Abkhazia

Table 2. Fst pairwise (below) and P(above) values between anchovy populations for RFLP data

	AZ*	GEO*	TWIN*	TSUM*	MAR*	ABK*
AZ	0.00000	0.00000	0.22523	0.00000	0.00000	0.00000
GEO	0.00858	0.00000	0.00000	0.00000	0.00000	0.00000
TWIN	0.21761	0.11473	0.00000	0.00000	0.00921	0.00000
TSUM	0.20208	0.14961	0.15949	0.00000	0.00000	0.00000
MAR	0.47754	0.42323	0.38916	0.21090	0.00000	0.00000
ABK	0.14650	0.14969	0.32329	0.15589	0.28684	0.00000

***P<0.001, ** P≤0.01, * P<0.05. AZ:Azov Sea; GEO:Georgia; TWIN:Trabzon winter; TSUM:Trabzon summer; MAR:Marmara Sea; ABK: Abkhazia

Table 3. Distribution of MtDNA d-loop haplotype among anchovy population Sixty-four composite genotypes (haplotypes-denoted with capital letters) based on RFLP digests of ten restriction enzymes (Hpa II, Hinf I, Hae III, Alu I, Ava II, Nci I RsaI, TaqI, Tru91, Hsp9211) in Control Region; relative frequencies of haplotypes per population, sample size (N), number of haplotypes (A), private haplotypes (P), effective number of haplotypes (N_e), haplotypic richness (R_h), genetic diversity (H_e),genotypic richness (R) and Tajima's D index (ns = non-significant)

	Azov Sea	Georgia	Trabzon Winter	Marmara Sea	Trabzon Summer	Abkhazıa
N	32	31	21	30	38	39
A	12	12	11	8	26	15
P	5	3	6	7	20	9
N_e	6.095	7.567	7.475	3.879	20.629	10.208
R_h	8.462	9.063	10.000	5.778	15.928	10.502
H_e	0.863	0.897	0.910	0.768	0.977	0.926
R=G/N	0.375	0.387	0.524	0.267	0.684	0.385
Tajima's D	-1.75465	-0.09590	-0.35048	-0.30121	-0.62556	0.76076
p-value	0.01600	0.52300	0.42000	0.42300	0.34550	0.22482

(P<0.001)

Table 4. Analysis of molecular variance (AMOVA) among and within populations. FST index represented the sum of variation among populations and variation within populations divided by total variation

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation Index
Among populations	5	101.336	0.57981 Va	23.03	FST=0.23027***
Within populations	185	358.559	1.93816 Vb	76.97	
Total	190	459.895	2.51796		

(***P<0.001)

divisions than other coastal pelagic. The main reason for these subdivisions is the fact that the Bosphorus Strait has a potential barrier for gene flow. This barrier along with ecological condition cause high haplotype frequencies in the Black Sea and the Marmara Sea. The European anchovy that be represented with two sub-divisions in the Black Sea is developed various migration scenarios due to migration characteristic between coasts of several countries as a sharing-stock (Ivanova *et al*. 2013; Gordina, Nikolskiy, Niermann, Bingel, & Subbotin, 1997). This study aims to reveal anchovy's mixed-stock forming areas in the Black Sea along with migration route and migration time of the populations of Azov, Marmara and Black Sea. The variation of distribution areas increases migration scenarios and caused arising of different sub-divisions.

The Marmara Sea anchovy shows a notable split from the Eastern Black Sea, the Aegean and the Mediterranean Sea anchovy in terms of meristic and biological properties. Whereas, there is mingling between the Marmara and neighbouring population from the Western Black Sea (Erdoğan, *et al*., 2009). On the other hand, anchovy population in the Eastern Black Sea seems to genetically vary from other regions. Turan (2004) reported morphometric differences between the Black and the Aegean Sea by investigating the anchovy species morphometrically and meristicly in Turkish waters. According to Dobrovolov (1987, 1992) there is a short genetic deviation D (Nei) between the Marmara and the Black Sea anchovy. Anchovy population of the Marmara Sea with Fst values differs from the anchovy populations of the Azov Sea and the Eastern Black Sea region genetically according to our study in overwintering season. It is fact that, anchovy populations of the Marmara Sea are isolated and become distinctive likewise the Azov Sea populations

Table 5. In principal coordinate analysis (PCoA) percentage of variation of mtDNA D loop region according to first three axes of 6 populations

Axis	1	2	3
%	26.99	69.16	3.85
Cum %	96.15	69.16	100.00
EigenValue	0.001	0.003	0.000

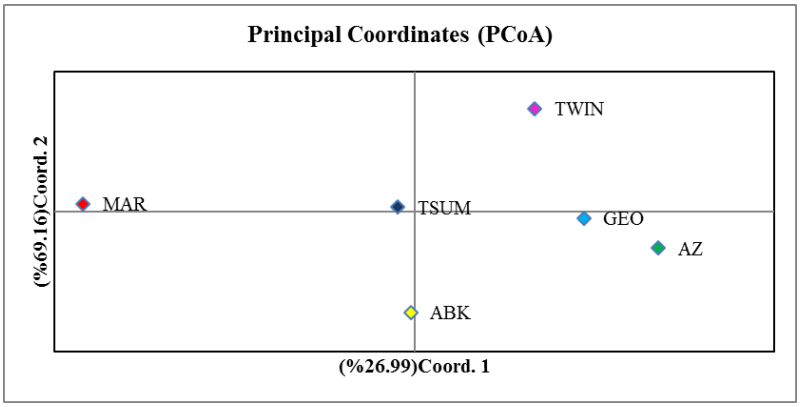

Figure 2. Principal Coordinates Analysis (PCoA) of anchovy populations. The amount of variation explained by each axis is 69.16 % for Coordinate 1 and 26.99% for Coordinate 2.

The Black Sea anchovy can be found in the Marmara Sea. Researchers stated that the Black Sea anchovy migrated into the Marmara Sea from fall to overwinter, and migrated back into the Black Sea for feeding and spawning in spring (Danilevsky, 1961). However, according to the current study genetic diversity within the same population is very low in Marmara group. So, it can be noted that, even if the Black Sea anchovy comes to the Marmara Sea for spawning, it differs from the Marmara Sea anchovy in the area it uses for breeding.

In summer, the Azov and Marmara Sea anchovies are found in the coasts of the Black Sea, Turkey (Gordina, 1997; Ivanova, et.al., 2013). Gordina (1997) found eggs of the Marmara Sea anchovy in the Black Sea and stated that the Marmara Sea anchovy is migrates to the Black Sea for reproduction in summer. Likewise, our study showed that haploids of the Marmara Sea anchovy exist in the Black Sea in summer season. Thus, the migration of the Marmara Sea anchovy was proved genetically.

Besides, Ivanova *et al* (2013) reported mixed population of the Azov and Black Sea anchovy lay eggs in April and even in July along the Varna coasts and the middle of the coast of the Black Sea, Turkey (Sinop). According to our results, summer samples of the Eastern Black Sea (Trabzon) includes haplotypes of Azov anchovy populations. With this output, existence of the Azov anchovy was proved in the Eastern Black Sea coasts of Turkey for the first time (Figure 3). Thus, determination of specific haplotypes living in the Marmara and Azov Sea in summer reveals that the Azov and Marmara Sea anchovy populations immigrate to the Eastern Black Sea coastal area during summer. In sum, the reproductive migration of the Azov and Marmara Sea anchovies to the Eastern Black Sea coasts were established genetically. Considering these, the migration routes of the Azov and Marmara Sea anchovy can be updated.

The Azov anchovy wasn't found only in the summer period in the Black Sea. It was reported that the Azov anchovy migrated southwards to Poti-Batumi in 1976-77, 1979, and 1983-85 (Chaschchin, 1996). It seems that the Azov anchovy population and the Black Sea anchovy population mixed in Batumi region. The Azov anchovy population approaches the Turkey-Georgia borders, and migrates southwards along the coastline down to Turkish waters in certain periods of time. On the other hand, it shows that the Black Sea anchovy is wintering into the Black Sea, but small amount of anchovy population moves from the west or south-west of the Georgian coast (Chaschchin, 1996). This study reveals that samples belonging to the Georgia population with Fts values featured both Black Sea haplotypes and Azov haplotypes. According to this result, it can be understood that the Black Sea and Azov Sea populations or mingled populations use the Georgia as a common area for spawning and feeding.

In conclusion, we registered that the Azov anchovy population moved to the Georgia coast in fishing season and moved to Turkish water in summer. Also we defined that the Marmara Sea anchovy population clearly separated from other populations but individuals of the Marmara Sea anchovy population was observed summer sampling period in

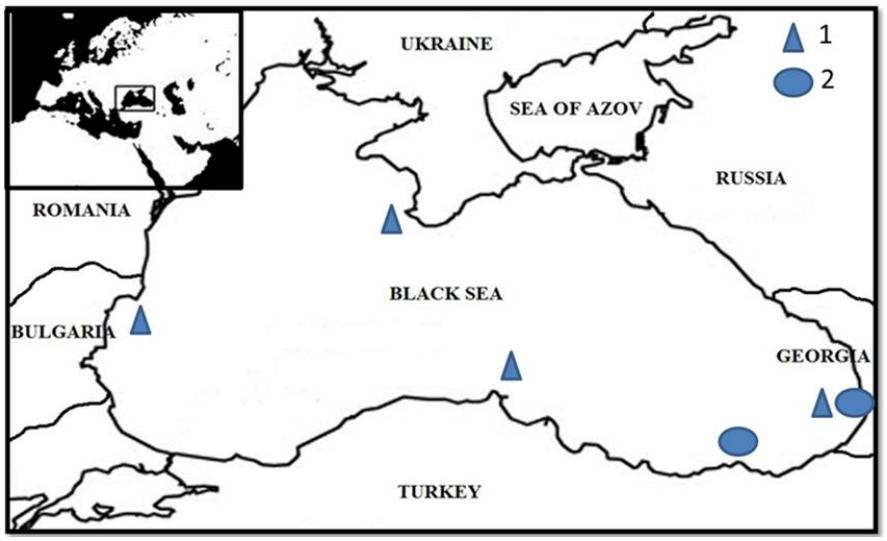

Figure 3. 1, According to Ivanova, et.al., (2013) Azak and Black Sea anchovy places mixed populations locations; 2, According to our study, Azak, Black Sea and Marmara Sea anchovy places mixed populations locations

the south-east Black Sea coast. In conclusion, it can be said that the Azov and Marmara Sea anchovy migrates for feeding only and this migration cause no gene flow. According to Tajima's d test results, anchovy populations show not strong selection. Despite this research have similarities with previous studies, it has proved many hypotheses genetically. With further studies and much more stations, we may have more information about the current situation of the Azov and Marmara Sea anchovy population in the Black Sea during summer period.

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