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Genetic Structure of Anchovy *Engraulis encrasicolus* in the Adriatic Sea using Microsatellite DNA Analysis

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Abstract

Stock structure analysis of anchovy from the Rovinj, Maslenica and Island of Korčulain in the Adriatic Sea was carried out by using 13 microsatellite loci. Overall, 259 alleles were detected in 13 loci, the number of alleles per locus and population ranged from 4 to 28. The allelic richness of overall loci was found to be highest in the Rovinj population and lowest in the Island of Korčula population. The highest and lowest value of population specific alleles was found in the Rovinj population and Island of Korčula population, respectively. Observed heterozygosity per population was ranged from 0.714 (Rovinj) to 0.678 (Maslenica). Pairwise F_{ST} values revealed that there is no significant genetic differences between populations (P>0.05). However, the highest and lowest genetic distance were found between the Rovinj and Maslenica populations (F_{ST}=0.00626) and between the Island of Korčula and Maslenica populations, respectively. The UPGMA dendrogram clustered the Maslenica and Island of Korčula populations together, and the Rovinj population was a distinct cluster from these two.

Keywords:
Anchovy, *Engraulis encrasicolus*, the Adriatic Sea, population genetics, microsatellite

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Introduction

European anchovy, *Engraulis encrasicolus* L. 1758, is widely distributed in pelagic waters in the Eastern Atlantic from the North Sea to South Africa, including the Mediterranean and Black Sea (Fischer et al., 1987; Turan et al. 2007). Currently, anchovy is one of the main target species for commercial fisheries in Europe, and most of its stocks are overfished at present. The high fishing pressure on anchovy requires the establishment of scientifically sound sustainable management plan(s) for their commercial fishery. One of the most important requirements for a sustainable management of a fishery is fishery is to understand how populations are partitioned: as a single unit or as several genetically distinctive groups with specific biological characteristics.

The European anchovy populations have been studied many times by means of morphological data (Bembo et al., 1996a; Turan et al., 2004, Aka et. al., 2004), allozymes (Bembo et al., 1996b; Borsa, 2002; Erdogan et al., 2009) and mitochondrial DNA (Magoulas et al., 1996; Grant, 2005; Magoulas et al., 2006) to elucidate their population structuring from several different areas. Based on these studies, the North East Atlantic and Mediterranean populations are partitioned today into several different spawning groups, isolated from each other by complex shorelines and oceanic regimes. For example, Pasteur and Berrebi (1985) detected allozyme-frequency differences between anchovy populations in different habitats (open sea, brackish lagoon) in the Golfe-du-Lion. The results from the study of Zarraonaindia et al. (2009, 2012) demonstrated hierarchical genetic differences between the European anchovy populations based on the variations in temporal and spatial scales. Their results also revealed slight genetic differentiation between the two major groups (or populations) of European anchovy, associated with different oceanic systems. Furthermore, mtDNA and microsatellite results in Borell et al. (2012) suggested the presence of at least three genetically differentiated groups: the West Cantabrian sea, the rest of the populations in the Bay of Biscay, and the Mediterranean.

Various molecular genetic techniques have been employed to describe fish stocks and species (Turan et al. 2009, Glover et al. 2011, Lamichhaneya et al. 2012). Microsatellite loci analysis have been most frequently used to delineate the different stocks of many exploited marine fish species (Shaw et al. 1999, Vinas et al. 2004, Glover et al. 2011) as well as anchovy (Borell et al. 2012, Zarraonaindia et al., 2012).

The present study investigated the genetic structure of anchovy stocks (or populations) along the Crotian coast of Adriatic Sea by using 13 microsatellite loci which have not been employed for these stocks previously.

Material and Methods

Sampling

Anchovy samples were collected from 3 different locations South Central (around the Island of Korčula), Central (Maslenica area), and North (near town of Rovinj)) along the Crotian coast of Adriatic Sea (Fig. 1). Anchovy specimens were collected via purse seiners and the whole collection

process was completed within the shortest possible time frame to avoid mixing of stocks due to migration or to obtain the same year cohort from all locations (Table 1). Tissue samples taken from 89 specimens were preserved in 95% ethanol until total genomic DNA extraction.

Figure 1. Map of sampling locations. Black circles indicate sampling point.

Table 1. Sampling locations, time and mean (±SD, N=32) weight and standard length of anchovy samples.

Sample Sites	Collection Time	Mean STL(SD)	Weight (g)
Island of Korčula	23 March 2012	165 (0.52)	29.0 (2.96)
Rovinj	25 March 2012	125 (0.55)	10.2 (1.35)
Maslenica	30 March 2012	102 (0.60)	5.6 (1.83)

Table 2. Primers and PCR conditions of 13 microsatellite loci used in this study.

Locus	Primers Sequences	Repeat motif	Allele range	Multiplex a
Ee2*	F: TCGCTAGGACGCTTTACGAC R: CCGGAGGTTCAGTGTTCATT	(CA)4GA(CA)12	244-278	I_{55}°c
Ee10*	F: GGTGGATGAAGTGGCAATCT R:CTGGGGTGGCATAACTGAAG	[(GT)9CT]2[(GT)2CT]3	190-270	I_{55}°c
Ee2-91a**	F: AGAGCAGGTTCTTGCTGTGG R: TGTGGTGCGCTACTATCAGG	(AGG)12	244-283	II_{55}°c
Ee2-483b**	F:ATGAGAAGGAGGACGGTGTG R: AATGGGATAGCTCGTTGTGC	(AGG)11	169-237	II_{55}°c
Ee2-508**	F: CACATGCTCGCTAAACATTG R: ACCTGATGCTGCTTGGTAGC	(AGG)8	154-191	II_{55}°c
Ee2-452a**	F: CCCAACCCTAGGGAGACATC R: TCGTTCAGCAAGCATACACC	(AC)13	254-290	III_{60}°c
Ee2-507**	F: GGAAGGGACCTAGATGGAGTG R: ATCCCATTGATGTCCTGAGC	(GAAA)14GAAC(GAAA)6GAGA (GAAA)4	231-358	III_{60}°c
Ee5-376**	F: CCACACCTACGGTGAGTGAC R: GCAAAGAGAATAGCACATGCAG	(TG)11	168-268	III_{60}°c
Ee2-135**	F: AGGGCAGTGACAGGAGAGTC R: TCGTTACCCTGCGTTTATACTG	(ATTAG)10	110-160	IV_{55}°c
Ee2-407**	F: AGGAATCTCCTTCCCGTCTC R: GTGGGTCTGTGGGTGTTTTG	(CA)13	147-197	IV_{55}°c
Ee2-477**	F: TTGGTGAGGAAGCAACAGTG R: TAAGATGGCACGCTGACTTG	(AGG)7	196-332	IV_{55}°c
Ee2-91b**	F: GGTCTTGAGCTTGGCATAGG R. CCGGAAGACACTCTGCACAC	(GT)5[(GT)4G]2(GC)2(GT)5	107-167	V_{60}°c
Ee2-165b**	F: GGGTGGGTTAAAGATGAAGC R: AGGGATCTTCAGGGAACCAG	(GT)14(GC)6(GT)9	187-206	V_{60}°c

* according to Landi et al., 2005, ** according to Pakaki et. al., 2009, a = Annealing temperature

PCR was performed in a 25 ml mixture of 50 ng template DNA, 1x PCR buffer, 1.5 mM $MgCl_2$ in the 2-plex I and the 3-plex II-IV, 2 mM $MgCl_2$ in the3-plex III and the 2-plex V, 0.2 mM dNTPs, 0.5 uM each primer, and 1 U *Taq* DNA polymerase. The amplification conditions were 3 min at 94 ˚C, 30 s at 94 ˚C, 1 min annealing (Table 1), 30 s at 72 ˚C, and 3 min at 72 ˚C, for 35 cycles at the 2-plex I, 2 min at 94 ˚C, 2 min at 94 ˚C, 45s annealing (Table 1), 45 s at 72 ˚C, and finally 10 min at 72 ˚C, for 35 cycles at 3-plex II-III-IV and 2-plex V. PCR products of microsatellites are

analysed in ABI 3730 automated DNA analyser (Macrogen Inc., South Korea). Microsatellite genotype of each specimen was scored by using GeneMarker software v 2.2.0 (SoftGenetics LLC, State Collage, USA).

Statistical analysis

Allele numbers in each locus and each population, observed and expected heterozygosities (*Ho, He*), pairwise F_{ST} values were calculated, and deviations from Hardy-Weinberg equilibrium (HWE) was tested with Fisher exact test, Markov chain algorithm (Guo and Thomson, 1992) was performed by using Arlequin ver. 3.5.1.3 (Excoffier, 2010). In order to determine population structuring, hierarchical analysis of molecular variance (AMOVA) was carried out, and genetic differentiation between populations were tested with infinite allele mutational (IAM) model by using Arlequin software. Allelic richness and F_{IS} (Weir and Cockerham, 1984) values between populations were calculated by FSTAT v 2.9.3.2 (Goudet, 2001). The GenAlEx v 6.5 software (Peakall and Smouse, 2012) was used to determine private alleles for each population.

Genetic variation between populations was determined based on Nei's genetic distance (Nei, 1978) by using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). For this aim, Tools for Population Genetic Analyses (TFPGA) v 1.3 (Miller, 1997) was used to create a UPGMA three with 10000-permutation bootstrap value per branch.

Results

In this study, 13 microsatellite loci in 89 specimens were analysed for determination of genetic structuring between the three Adriatic anchovy populations. Overall, 259 alleles were detected in 13 microsatellite loci. The number of alleles per locus and per population ranged between 4 (4 at Ee2-165b in the Maslenica population) and 28 (28 at Ee5-376 in the Maslenica population). Overall allelic richness was highest in the Rovinj population and lowest in the Maslenica population. Only 2 out of 13 loci (Ee2-453a and Ee2-165b) did not show significant deviations from the Hardy-Weinberg Equilibrium (HWE) in all three populations, while Ee2-91a, Ee2-452a, Ee2-507, Ee2-407, Ee2-91b and Ee2-165b loci showed no significant deviations from the HWE when only all 3 populations were combined together. Only loci Ee2-453a and Ee2-165b were found to be in Hardy Weinberg equilibrium.

The highest value of population specific alleles was found in the population of Rovinj, the lowest was in the population of Island of Korčula. Observed heterozygosity per population ranged from 0.714 (in the Rovinj population) to 0.678 (in the Maslenica population) while expected heterozygosity per population ranged from 0.810 (in the Maslenica population) to 0.823 (in the Island of Korčula population) (Table 3).

Table 3. Statistics of 13 microsatellite loci analysed.

Locus	Populations			
	Island of Korčula	Rovinj	Maslenica	All
Ee2				
N	27	29	30	86
NA	11	8	11	14
AR	11,000	7,793	10,490	10,366
Ho	0,481	0,448	0,433	0,453
He	0,695	0,647	0,672	0,670
F_{IS}	**0,312**	**0,311**	**0,359***	**0,325***
p (HW)	0,080	0,009	0,000	0,000
Ee10				
N	28	29	32	89
NA	11	16	14	27
AR	10,926	15,502	13,127	15,257
Ho	0,714	0,793	0,181	0,764
He	0,806	0,812	0,820	0,813
F_{IS}	0,115	0,024	0,048	0,060
p (HW)	0,040	0,010	0,254	0,010
Ee2-483b				
N	28	29	32	89
NA	8	12	9	14
AR	7,929	11,786	8,528	10,165
Ho	0,679	0,759	0,625	0,685
He	0,831	0,872	0,751	0,820
F_{IS}	**0,186**	0,132	0,171	**0,165**

p (HW)	0,126	0,543	0,012	0,007
Ee2-508				
N	28	29	32	89
NA	6	9	10	14
AR	5,964	8,862	9,349	8,841
Ho	0,357	0,517	0,500	0,461
He	0,651	0,694	0,709	0,698
F_{IS}	**0,456***	**0,258**	**0,298**	**0,341***
p (HW)	0,000	0,030	0,012	0,000
Ee2-91a				
N	28	29	32	89
NA	11	12	14	16
AR	10,963	11,717	13,636	12,345
Ho	0,786	0,828	0,781	0,798
He	0,882	0,864	0,917	0,890
F_{IS}	0,111	0,043	0,150	**0,105**
p (HW)	0,124	0,529	0,002	0,101
Ee5-376				
N	28	29	32	89
NA	23	20	28	43
AR	22,532	19,234	25,401	22,507
Ho	0,464	0,552	0,563	0,528
He	0,900	0,909	0,943	0,918
F_{IS}	**0,489***	**0,397***	**0,408***	**0,426***
p (HW)	0,000	0,000	0,000	0,000
Ee2-452a				

N	28	29	32	89
NA	17	16	16	19
AR	16,819	15,637	15,324	15,468
Ho	0,857	0,897	0,938	0,899
He	0,932	0,910	0,926	0,925
F_{IS}	0,082	0,016	-0,013	0,028
p (HW)	0,370	0,875	0,803	0,167
Ee2-507				
N	27	29	32	88
NA	26	27	27	43
AR	26,000	26,157	24,714	27,550
Ho	1,000	0,862	0,938	0,931
He	0,963	0,966	0,954	0,966
F_{IS}	-0,038	**0,109**	0,017	0,035
p (HW)	0,571	0,023	0,457	0,189
Ee2-407				
N	28	29	32	89
NA	16	11	16	20
AR	15,783	10,789	14,724	14,724
Ho	0,679	0,759	0,688	0,708
He	0,877	0,812	0,840	0,843
F_{IS}	**0,229**	0,067	**0,184**	**0,161***
p (HW)	0,007	0,213	0,053	0,062
Ee2-135				
N	28	29	32	89
NA	12	14	12	16

AR	11,928	13,851	11,640	12,927
Ho	0,821	0,793	0,781	0,798
He	0,900	0,907	0,886	0,896
F_{IS}	0,085	**0,128**	0,120	**0,110**
p (HW)	0,101	0,047	0,000	0,000
Ee2-477				
N	28	29	32	89
NA	10	11	7	15
AR	9,857	10,848	6,844	9,504
Ho	0,750	0,586	0,688	0,674
He	0,850	0,849	0,822	0,836
F_{IS}	0,118	**0,314***	**0,166**	**0,194***
p (HW)	0,281	0,004	0,107	0,001
Ee2-91b				
N	27	29	32	88
NA	10	8	8	11
AR	10,000	7,927	7,665	8,416
Ho	0,778	0,931	0,625	0,772
He	0,860	0,819	0,770	0,818
F_{IS}	0,095	-0,139	**0,191**	0,056
p (HW)	0,449	0,242	0,020	0,487
Ee2-165b				
N	27	29	32	88
NA	6	6	4	7
AR	6,000	5,924	3,997	5,276
Ho	0,481	0,552	0,469	0,500

He	0,564	0,621	0,516	0,564
F_{IS}	0,150	0,114	0,094	0,113
p (HW)	0,280	0,490	0,508	0,571
All				
N	28	29	32	89
NA	12,846	13,077	13,538	-
AR	12,746	12,771	11,953	-
Ho	0,681	0,714	0,678	-
He	0,823	0,822	0,810	-
F_{IS}	0,176	0,134	0,166	-
PA	2,385	2,846	2,769	-

N = Sample size, NA = Number of alleles, AR = Allelic richness, *Ho* = observed heterozygosity, *He* = expected heterozygosity, *p*(HW) = *p* value of Hardy-Weinberg equilibrium, F_{IS}= the average deviation from Hardy-Weinberg proportions in subpopulations, PA = Private alleles.

Pairwise F_{ST} values showed that three populations has no significant differentiation from each other upon to *P* values ($P>0.05$). However, the highest genetic distance was found between Rovinj and Maslenica populations ($F_{ST}=0.00626$), the lowest was between the Island of Korčula and Rovinj populations (Table 4).

Table 4. Pairwise F_{ST} estimates of genetic differentiation based on infinite allele mutational (IAM) model (F_{ST}).

	Island of Korčula	Rovinj	Maslenica
Island of Korčula	-		
Rovinj	0.00129	-	
Maslenica	0.00250	0.00626	-

The UPGMA dendrogram revealed phylogeographic relationship of populations and the clustered the Maslenica and Island of Korčula populations together while the Rovinj population was a highly distinctly clustered from them (Figure 2).

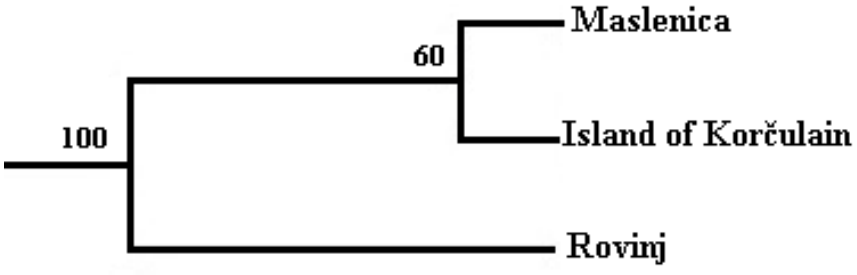

Figure 2. UPGMA dendrogram shows genetic relationship between the populations. Bootstrap values of 10.000 replications are indicated on nodes in percentages.

Discussion

The present microsatellite study revealed no significant genetic heterogeneity among anchovy populations from three different localities in the Adriatic Sea. Interestingly, there were clear size and weight differences of a year cohort sampled from three different latitudes in the Adriatic Sea. The observed growth rate increase of anchovy from north to south Adriatic Sea could be attributed to different environmental conditions of their spawning grounds and nursery areas. These morphologic differences may be attributed to that anchovies in the Rovinj, Maslenica and Island of Korčula regions originated from different spawning grounds, and faced with different water columns and nutrient-rich habitats in their nursery areas. Tudela (1999) reported morphological differences against a background of genetic homogeneity among anchovy samples from the North Western Mediterranean and suggested that the environment was the main determinant of morphological variation among anchovy populations. The geographical limit between stocks, as delineated by a drop in electromorph frequency for locus IDHP-2, was coincident with a change in hydrology between shallow (<50m) waters of the northern Adriatic and the deep sea waters of the southern-central Adriatic (Bembo et al., 1996a).

Although the northern Adriatic region is in geographic continuity with the open sea, as a shallow and semi-enclosed marine area it is subjected to a sub-tropical climate cycle and to a large runoff, which make the dynamics of its ecosystem the most strongly depending by air–land–sea interactions in the whole Mediterranean, making the North Adriatic as a region where ongoing climate changes are expected to have significant consequences on the marine environment (Malone et al., 1999). Comparing the Adriatic anchovy with the other Mediterranean anchovies, Borsa (2002) suggested that the south-central Adriatic stock is genetically similar to geographically distant western Mediterranean and Gulf of Biscay populations, whereas the northern Adriatic stock is genetically similar to anchovies in the brackish lagoons of the Golfe-du-Lion. Hence, the emerging result from Borsa's (2002) research was that *Engraulis encrasicolus* appears to consist of at least two biological species (Group I, Group II) which are both present in the Golfe-du-Lion and in the Adriatic Sea.

On the other hand, several researchers have reported differences between populations of anchovies in the Adriatic Sea. Bembo et al. (1995) detected heterogeneity in mtDNA haplotype frequencies among anchovy samples taken around the Italian Peninsula. Later, they (Bembo et al.,

1996a) detected significant differences between a putative south-central and northern anchovy stocks of the Adriatic Sea which were sampled at regular intervals over a two-year period. Further comparison of the allozyme frequency data between those samples suggested the existence of separate and temporally stable stocks of the Adriatic anchovy (Bembo et al.,1996a), which was also supported by Carvalho and Hauser (1998). Although all these studies supports the existence of more than one stock, fishery legislations in action are based on the presumption of a single anchovy stock in the Adriatic Sea (Cingolani et al., 2004).

Genetic structuring analyses (Landi et al., 2005, Pakaki et al., 2009, Borrell et al., 2012) performed for the Adriatic anchovy populations demonstrated that the size of alleles for all microsatellite loci ranged from 107(EE2-91b) to 358 (EE2-507). Allel sizes in these study showed an overlap to the other studies (Landi et al., 2005, Pakaki et al., 2009, Borrell et al., 2012) except for EE2-91a locus. Allel sizes of EE2-91a locus were reported to be in between 205 to 256 in an earlier study (Pakaki et al., 2009), but it ranged in between 244-283 in this study.

When Borrell et al (2012) screened the populations from 5 different locations of the Gulf of Biscay and from one location of the Adriatic Sea in 14 microsatellite loci, he found that average allelic richness and observed/expected heterozygosity (H_o/H_e) were 8:39 and 0.658/0.786 respectively. Borrell et al. (2012) found no significant genetic differences when he included all 14 loci into analyses, but he detected a significant genetic differentiation (F_{ST}=0.014, P=0.0033) within the Gulf of Biscay in 5 loci.

F_{ST} values in the present study ranged in between 0.00129 to 0.00626 and were found similar to that of Borrell et al. (2012) which ranged in between 0 to 0.0006. This indicates that there are no restrictions for migration of population among the different areas of the Adriatic Sea. The marine species usually show low genetic differentiation due to lack of major geographical barriers to dispersal and gene flow (Ward et al., 1994). Marine species with high dispersal and large population size such as pelagic fish often result in low or no genetic structuring across large geographic scales.

In conclusion, even though, growth of the same year anchovy cohorts clearly varied with their latitudinal distribution along to Crotian coast of the Adriatic Sea, they did not show any evidence of genetic differentiation in 13 microsatellite loci screened in this study. Nevertheless, this do not exclude the possibility that the other DNA markers such as mitochondrial 16S rDNA or different loci of microsatellites (Erguden et al., 2010; Zarraonaindia et al., 2012) could be more used in detecting genetic differences within anchovy stock(s) of the Adriatic Sea.

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