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Mitochondrial DNA Analysis of the European Anchovy in the Southern Mediterranean and Northern Atlantic Coasts

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Abstract: The present study was undertaken to elucidate the genetic structure of anchovy populations in the region of the Straits of Gibraltar. Five natural populations: Punta Umbria, Barbate and Almeria (South of Spain) and Larache and Nador (North of Morocco) were studied by the analysis of the RFLPs of the complex NADH dehydrogenase gene (*ND* gene). Genes were amplified with universal primers and the PCR product obtained showed a size of 2.5 Kb coding for genes *ND 5/6*. The haplotype diversities ranged from 0.6846 to 0.8987 with variation of 83.3% within populations and 16.97% between populations. Genetic variability was found to increase from the Atlantic to the Mediterranean Sea in the Spanish populations, but it decreased in the Moroccan populations. Genetic distance relationships among populations showed a geographic structure grouping the 5 populations in three clusters: one contained two of the Spanish populations, another contained the two Moroccan ones and the third contained the other Spanish population. This was located between the others in the Strait of Gibraltar. This genetic structure could be due to several factors including gene flow, depending on the hydrology of the zone, biology of the anchovy and habitat. These data are of interest for the design and application of management and conservation strategies for the genetic resources of *E. encrasicolus* in the area.

Key words: PCR, *Engraulis encrasicolus*, mtDNA, RFLPs, NADH dehydrogenase, *ND* gene

INTRODUCTION

The European anchovy *Engraulis encrasicolus* is a small pelagic fish, which lives in large shoals at depths of more than 100 m in open sea and coastal zones. It is an euryhaline species inhabiting waters of very varied salinity which accounts for its wide distribution (Magoulas *et al*., 1996).

The anchovy is distributed from 62° N, to 19° S including the coasts of the Atlantic from Bergen in Norway, to Capetown in South Africa and in the Mediterranean Sea, the Black Sea and Sea of Azov. This species presents a short life cycle of 3 to 4 years and fast growth, reaching first sexual maturity at the end of the first year of life. Reproduction takes place between the months of July and September.

The clupeiform genus includes eight species, three of which are commercially important: the European anchovy, *Engraulis encrasicolus*, the Japanese anchovy, *E. japonicus* and the Peruvian anchoveta *E. ringens* (Inoue *et al*., 2001). Achovy are found in large abundance in the Mediterranean Sea, associated with zones of local upwelling of waters rich in nutrients (Bakun and Agostini, 2001). Anchovies support the largest fisheries in the world, with millions of tons harvested annually although

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Table 1: Captures in tons of European anchovy *Engraulis encrasicolus* of years 1981, 1995 and 2004 (Statistics of FAO) of Morocco and Spain

	Mediterranean			Eastern central atlantic		
Years	1981	1995	2004	1981	1995	2004
Morocco	19.533	691	389	2.702	10.489	7069
Spain	38.722	17.000	8.213	200	-	-

a significant reduction of the catch of *Engraulis encrasicolus* has occurred in the last two decades (Table 1). Lately, this reduction in catches has been the reason for several seasons of prohibition of fishing along the Spanish coasts of both the Atlantic (Northern part of the Peninsula) and the Mediterranean.

Magoulas *et al*. (1996, 2006) studied RFLPs of the whole mtDNA of the anchovy and proposed that the species entered the Mediterranean from the Atlantic about 5 million years ago when the Strait of Gibraltar opened and that the current hydrographic forces contributed to the dynamic maintenance of the mitotypic diversities originated from these geologic events. General studies have reported population differences within and between Black Sea and Azov Sea anchovies.

Two forms, one of the inshore habitat and the other of the open sea habitat, have been identified within *Engraulis encrasicolus* in Europe, on the basis of both its morphology and nuclear DNA markers. *E. encrasicolus* refers to the species living in the deep waters of the open-sea while *E. albidus* (a criptic species) refers to the species found closer in-shore and in the inland waters (Borsa, 2002). Several studies have demonstrated stock discrimination among the European anchovy obtained from the Seas around the Peninsulas of Italy and Greece, using PCR-amplified mitochondrial DNA analysis (Bembo *et al*., 1995) and allozymic and morphometric data (Bembo *et al*., 1996). In spite of this, Garcia *et al*. (1994) examined the same species but found no differences between the samples from Barcelona on the Spanish coast and the samples from Elbe Island on the Western Italian coast. Tudela *et al*. (1999) reported no significant genetic structure among Oceanic populations in the Northern part of the Western Mediterranean and suggested that the environment was the main cause of morphological variation among anchovy populations. Magoulas *et al*. (1996) studied the relationship between mitotypes of a population in the Bay of Biscay and other populations in the Black Sea, Northern Aegean and Eastern Mediterranean. In Magoulas *et al*. (2006) extended the geographic zone for his study of mitochondrial DNA analysis in European anchovy including the Mediterranean Sea to the Eastern Atlantic as far Southern Senegal. They found eighty-eight haplotypes grouped in two clades distributed in a mosaic patter with abrupt changes between some areas and gradients between other areas.

Hence, in this study we examine the genetic structure of stocks of the European anchovy in the area within and near the Strait, using mtDNA RFLPs of the *ND5/6* region. Lately, many studies have been undertaken on the mtDNA gene encoding for complex NADH dehydrogenase (the *ND* gene) to demonstrate genetic variability between several stocks of fish. Gene coding for subunits *ND5/6* of this complex have been used successfully in Clupeids (Bembo *et al*., 1995; Hauser *et al*., 1995), in Salmonids (Hansen and Loeschcke, 1996; Nielsen *et al*., 1998; Hansen *et al*., 1999; Sell and Spirkovski, 2004) and in Silurids (Agnese *et al*., 1997; Triantafyllidis *et al*., 1999; Krieg *et al*., 2000; Wang *et al*., 2004).

MATERIALS AND METHODS

The samples to be analyzed were ordered from the local communities of fishermen in order to avoid confusion among samples. The fish were caught from 5 natural populations, Punta Umbria, Barbate and Almeria on the Andalusian coast and Larache and Nador on the Moroccan coast (Fig. 1). Samples were collected during the year 2005 and each consisted of 40 individuals. The fish were conserved on dry ice prior to being transported to the laboratory. Once at the laboratory they were conserved at -80°C.

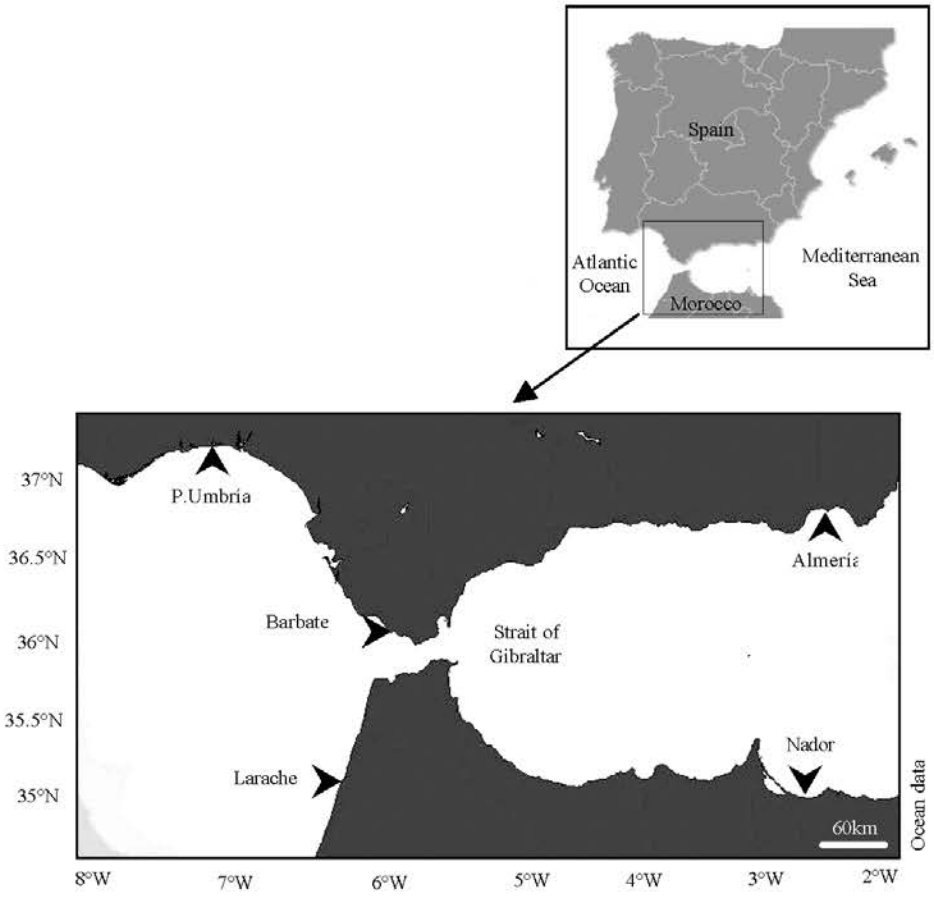

Fig. 1: Geographic location of the studied populations of *Engraulis encrasicolus*

mtDNA RFLP Analysis

Total genomic DNA was extracted from muscle using the FastPrep System and FastDNA Kit (Q. Bio gene). Universal vertebrate primer sequences were used to amplify mtDNA. The gene encoding for subunits of *ND5/6* encompassed the complete NADH-dehydrogenase (Cronin *et al.*, 1993). The primer sequences were:

5'- AAT AGT TTA TCC AGT TGG TCT TAG -3'
5'- TTA CAA CGA TGG TTT TTC ATA GTC A -3'

Polymerase Chain Reactions (PCR) were run using the PCR system 2700 GeneAmp® Thermal cycler. The reaction mixture (50 µL) contained 1 mM PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 25 pmol of each primer and 2 units of Euro-Taq DNA polymerase (Euro-Clone, Italy). The cycling conditions were 95°C for 5 min, 30 cycles (denaturation at 94°C for 30 sec, primer annealing at 49°C for 1 min 30 sec and primer extension at 72°C for 1 min 30 sec) and a final extension step (72°C for 10 min). Restriction digestions were performed on aliquots of 5 µL of PCR product. Resulting fragments were separated electrophoretically in 1 to 3% agarose gels stained with ethidium bromide. The following restriction enzymes were applied: *Hinf* I, *Cfr* 13I, *Hha* I and *Taq* I.

Data Analysis

Restriction enzyme digest haplotypes were scored according to the order of appearance during the analysis, with A being the first, B second, etc. Individuals were characterized by restriction site differences inferred from fragment variation. The RFLP scoring was conducted across all enzymes over the *ND5/6* fragment and then combined into a single composite haplotype. Each composite haplotype represented the inferred restriction site pattern for the 4 enzymes across the amplified mtDNA fragment and were represented by 4 letter codes, with each letter representing the RFLP for *Hinf* I, *Cfr* 13I, *Hha* I and *Taq* I, respectively. The data were analysed using various programs contained in the ARLEQUIN 3.01 (Excoffier *et al*., 2006) and PHYLIP 3.57c (Felsenstein, 1995) computer packages. The significance level was obtained by 10000 randomizations.

RESULTS

The size of the PCR-amplified fragment of mtDNA segment in *E. encrasicolus* was 2.5 Kb for *ND5/6*. The screening of all populations showed a total of 55 electrophoretic patterns after digestion with four restriction enzymes; these patterns could be grouped in 36 composite haplotypes numbered from 1 to 36 (Table 2). The restriction enzymes that showed polymorphic patterns, *Hinf* I, *Cfr* 13I,

Table 2: Engraulis encrasicolus mtDNA composite haplotypes and their distribution across 3 samples from Spain (Punta Umbria, Barbate and Almeria) and 2 samples from Morocco (Larache and Nador)

Haplotypes	P. Umbria	Barbate	Almeria	Larache	Nador
1 AAAA	19	10	8	20	22
2 AAAB	3	-	-	2	2
3 AAAC	-	-	5	1	-
4 AAAD	4	-	-	-	-
5 AAAF	-	-	3	-	-
6 AAAG	-	-	-	1	-
7 AABA	-	-	-	1	4
8 AABC	-	-	1	-	-
9 AAEA	-	-	-	2	-
10 AAGA	1	-	-	-	-
11 AAHA	-	-	-	-	3
12 ABAG	-	1	-	-	-
13 AFAA	-	-	-	-	1
14 BAAA	4	-	-	2	-
15 BBBH	-	-	1	-	-
16 CAAA	1	-	-	-	-
17 CADA	1	-	-	4	-
18 DAAA	4	5	-	-	-
19 DAAB	1	-	-	-	-
20 DACC	-	6	-	-	-
21 DAHB	1	-	-	-	-
22 DBAE	-	-	1	-	-
23 DBBC	-	5	8	2	3
24 DBBG	-	1	-	-	-
25 DBCC	1	3	2	4	2
26 DBCE	-	1	-	-	-
27 DBFB	-	1	1	-	-
28 DDBC	-	-	1	-	-
29 DEBC	-	-	-	-	1
30 DECC	-	3	-	-	-
31 DFBC	-	1	-	-	-
32 DFIC	-	1	-	-	-
33 EBBC	-	-	4	-	-
34 ECBC	-	1	1	-	2
35 FBFB	-	1	3	-	-
36 GAAA	-	-	1	1	-
h	0.7538	0.8897	0.8987	0.7359	0.6846
SE ±	0.0647	0.0276	0.0245	0.0701	0.0771
n	40	40	40	40	40
ui	05	06	06	02	03

(*h*) Haplotype diversity, (SE): Standard Error, (n): Sample Sizes, (ui): Unique Haplotypes

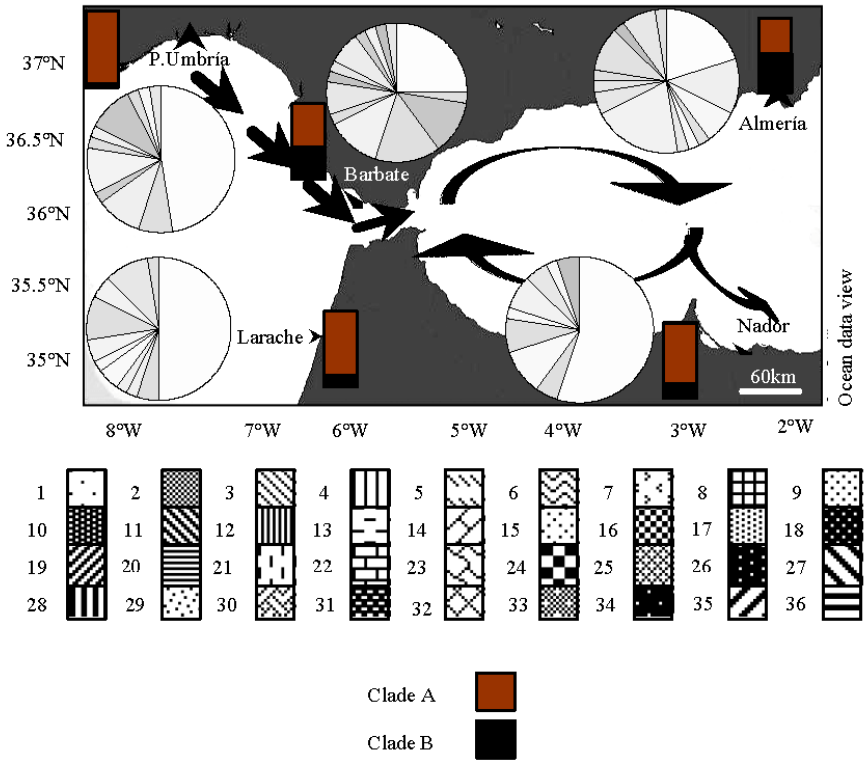

Fig. 2: Distribution of haplotype frequencies of five samples of *Engraulis encrasicolus*, 3 from Spain (Punta Umbria, Barbate and Almeria) and 2 from Morocco (Larache and Nador) and circulation of the surface water masses which are presented with the black arrows. The area of the circles is proportional to the haplotype frequency class. Shading refers to the different haplotypes. Colour column is proportional to clade frequencies: in brown clade A, in black clade B

Hha I and *Taq* I, were used to define haplotypes. Eight different restriction profiles were found for *Hha* I (A-H), 6 for *Hinf* I (A-F), 9 for *Cfr* 13I (A-I) and 8 for *Taq* I (A-H). 22 out of 36 haplotypes were encountered in a single fish (unique haplotypes) and 14 out of 36 haplotypes were widely distributed between individuals. The samples from Barbate and Almeria showed a large number of unique haplotypes (6 in each population) and haplotype 1 (AAAA) and 25 (DBCC) appeared in all samples (Table 2). According to the measures, the lowest level of variation was found in Nador samples, where 55% of the fish were of genotype 1 (Table 2 and Fig. 2).

Haplotype diversity (h) indicates the probability that any two randomly chosen haplotypes are distinct from one another. Most of the haplotype diversities for the samples are relatively high (Table 2) and ranged from 0.6846 to 0.8987, with sample mean haplotypes diversity of 0.7920. Most differences between haplotypes could be explained by the loss or gain of one restriction site. The topology of the minimum spanning tree obtained with the mtDNA composite haplotypes shows a clear dichotomy, with 19 haplotypes forming one clade named A and 17 forming another clade called B (Fig. 3). Composite haplotype AAAA is connected to all but 1 of 19 haplotypes by a single step and 2 other haplotypes by two steps. The high frequency of haplotype AAAA and the fact that it occupies the centre of a group A phylogeny suggest that AAAA is the most likely ancestral haplotype in this group. The group B of haplotypes is different, because there are 2 haplotypes which shares the centre of the phylogeny tree. The haplotype 23 (DBBC) which is most frequent and the DBCC which is more common and appears as the most likely ancestral haplotype in this clade. The analysis of

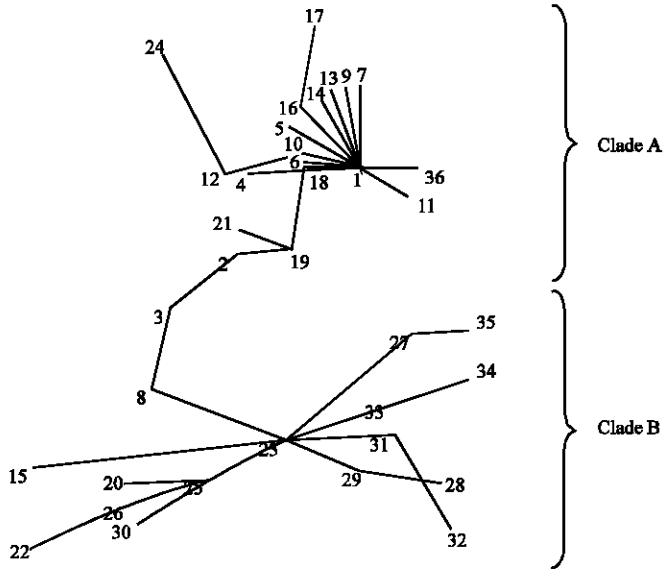

Fig. 3: Minimum spanning tree between *Engraulis encrasicolus* mtDNA composite haplotypes of 5 anchovies samples, 3 from Spain (Punta Umbria, Barbate and Almeria) and 2 from Morocco (Larache and Nador)

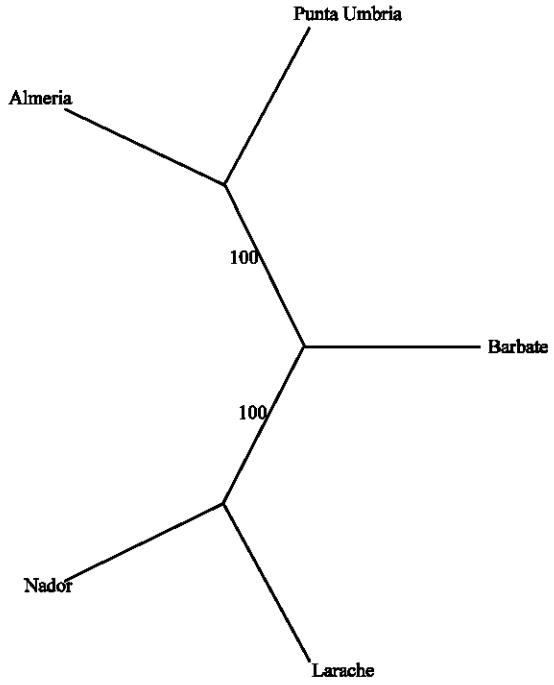

Fig. 4: Concensus tree of the five populations of European anchovy *Engraulis encrasicolus*, 3 from Spain (Punta Umbria, Barbate and Almeria) and 2 from Morocco (Larache and Nador)

Table 3: ANOVA showing the variation among and within 5 populations, 3 from Spain (Punta Umbria, Barbate and Almeria) and 2 from Morocco (Larache and Nador). (Excoffier *et al*., 1992)

Source of variation	df	Sum of squares	Variance components	Percentage of variation	F-value	p-value
Among population	4	118.220	0.6584	16.97	0.1697	<0.001***
Within population	195	628.025	3.2206	83.03		
Total	199	746.245	3.8790			

***p<0.001

Table 4: F_{ST} pairwise comparison of *ND5/6* haplotype frequencies among 5 anchovies samples, 3 from Spain (Punta Umbria, Barbate and Almeria) and 2 from Morocco (Larache and Nador). Markov chain length: 10000 steps. (Weir and Cockerham, 1984) with Bonferroni's corrections

Samples	P.Umbria	Barbate	Almeria	Larache
Barbate	0.3121***			
Almeria	0.3230***	0.0368		
Larache	0.0259	0.2033**	0.2147***	
Nador	0.0669	0.1615**	0.1693**	0.0081

*p<0.05, **p<0.01, ***p<0.001

geographic heterogeneity in *ND5/6* fragments of *E. encrasicolus* was carried out with 10000 permutations of the data set. A hierarchical analysis of molecular variance (AMOVA) revealed a significant differentiation among populations (F_{st} = 0.1697, p<0.001) (Table 3). In pairwise comparisons based on haplotype frequencies, the Almerian and Barbate anchovy's samples presented varying degrees of significant geographical differentiation from all other samples (Table 4). The Reynold's distance based on values of the haplotype divergence between the populations, also demonstrated a clear geographic structuring (Fig. 4).

DISCUSSION

The present study has revealed a high level of variability of the *ND5/6* fragment in the European anchovy *Engraulis encrasicolus* in an area of 1000 Km around the mouth of the Straits of Gibraltar, including both Mediterranean and Atlantic zones. We have obtained 36 composite haplotypes in 200 fish, with an average haplotype diversity of 0.79. Significant heterogeneity in mtDNA haplotype frequencies among anchovy populations across the Mediterranean has been reported (Bembo *et al*., 1996; Magoulas *et al*., 1996; Borsa 2002; Borsa *et al*., 2004). Bembo *et al*. (1995) analysed the same *ND5/6* region in *E. encrasicolus* in 140 fish with 6 restriction enzymes and they found 53 composite haplotypes and a mean haplotype diversity of 0.88. Hauser *et al*. (2001) studied the *ND3/4* and *ND5/6* regions of the Atlantic Herring *Clupea harengus* also finding a high degree of variability with haplotype diversity of 0.89 in 445 fish and concluded than the order clupeiformes presented high levels of mtDNA variation and that the *ND5/6* gene is more variable than *ND3/4*. In other fishes, a study of Chow *et al*. (1997) on swordfish in the Mediterranean showed a genotypic diversity lower than that found in this study, h = 0.70. A recent study on the *ND5/6* genes of the long-tailed hake *Macrurous megellanicus* has found low levels of genetic diversity with h = 0.61 in 160 fish. (D'Amato and Carvalho, 2005).

The test of randomization of Markov in the total matrix in pairs of data showed a significant increase of the genetic distance with the geographic distance in all population (Table 4), suggesting an effect of isolation by distance and habitat. Comparisons in pairs of samples of diverse populations formed three different clusters between the populations. Since the anchovies naturally group in large shoals of fish, a large number of females should be participating in the reproductive processes within the studied populations; in fact we found 36 composite haplotypes in 200 analysed individuals. However, the presence of a pattern of dominant haplotypes and numerous rare ones has been attributed to differential survival of fish, mainly during early stages of life, also to the high reproductive

success of a few females or to either a founder effect or a bottleneck followed by population expansion (D'Amato and Carvalho, 2005).

Genetic distance relationships among populations of *Engraulis encrasicolus* observed in the dendrogram of the *ND5/6* fragment (Fig. 4) showed a geographic structure grouping the 5 populations in three clusters: one contained two of the Spanish populations, another contained the two Moroccan ones and the third contained the other Spanish population. This was located between the others in the Strait of Gibraltar. The marine environment is usually considered as lacking major geographical barriers to dispersal and allowing gene flow to occur. Grant and Bowen (2006) proposed the inhabiting of populations of European anchovies in long shorelines would provide opportunities for range shifts during extreme climate oscillations. In addition the climate-induced selection could influence the distribution of genetic diversity and could thereby limit demographic inferences.

However, the amount and distribution of genetic diversity is caused not only by contemporary levels of gene flow, but also by demographic processes, population history and selection. In fact, anchovies are commonly considered highly mobile but their localised spawning behaviour or migratory patterns may result in restricted gene flow, especially among waters of contrasting hydrography (Bembo *et al*., 1996).

Magoulas *et al*. (2006) found that European anchovy show a remarkable degree of genetic population subdivision among areas, showing an haplotype diversity in the Mediterranean Sea of 0.8315 while in the Atlantic ocean the diversity was only 0.4717.

One fundamental difference between European anchovy and other coastal pelagic species, which show little genetic population subdivision, is that European anchovy inhabit coastal seas isolated from one another by peninsulas and narrow straits. The constriction of the Strait of Gibraltar partially isolates the Mediterranean from the Eastern North Atlantic. Many of the haplotype frequency discontinuities occur across these potential barriers to gene flow.

In general,present results demonstrated that genetic variability is increased from the Atlantic towards the Mediterranean Sea; this finding could be explained by the increase of gene flow depending on hydrology of the zone, so that the difference of the sea level and the atmospheric pressure between the Western and Eastern sectors of the Straits of Gibraltar promotes the entry of superficial Atlantic water towards the Mediterranean Sea, forming an anticyclonal turn of 220 to 300 m of depth (Perkins *et al*., 1990). Following the turn, water current is directed towards the West of Nador and separates in two branches, the first moving towards the East along the Algerian littoral and the second branch being directed to the West in the direction of the Strait of Gibraltar forming the Southern branch of the anticyclonic turn. This water movement could explain the decreased variability in the populations of the Moroccan zones (Fig. 2).

On the other hand, most of the natural fluctuation of fish populations is associated with recruitment, a complex process in which both environmental and biological factors take part. However, the control mechanisms of fish populations act before the individuals reach exploitation size; that it is, during the Ichthyoplankton phase, the first stages of fish development. In the case of the Strait of Gibraltar the peculiar offshore hydrodynamics set off a clear West-East displacement of ichthyoplankton, due to an inflowing Atlantic water-jet, moving adjacent waters in the same direction (Rubín and Feigl, 1997).

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