



Genetic population structure of the European anchovy (*Engraulis encrasicolus*) based on mitochondrial DNA sequences along the Moroccan coast

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Abstract. The European anchovy (*Engraulis encrasicolus*) is considered to have, in addition to its ecological role, an important economic value, it is regularly targeted by fishers in Morocco. In this study, we investigated the Clade frequencies and population structure of this species using a 682 bp segment of cytochrome b gene. A total of 138 individuals collected from four geographic areas in Morocco were analyzed. The results showed 118 haplotypes and 147 segregating sites (S), of which 59 sites were parsimoniously informative. Higher levels of genetic variation were found for the haplotype diversity $h = 0.9868 \pm 0.0061$, but a concatenated mean nucleotide diversity for all samples was 0.008946 ± 0.004740 . The most dominant clade of *E. encrasicolus* in Morocco was Clade A with a percentage ranging from 89% in Alboran Sea to 95% in the Atlantic coast. Overall, there was a significant genetic differentiation among the 4 Moroccan zones ($\Phi_{st} = 0.01283$; $p = 0.03910$). Pairwise Φ_{st} among populations revealed that the most genetic variation occurred between Alboran population and Atlantic populations, mainly when we use the sequences of the dominant clade (Clade A), while the genetic differentiation was low or in moderate level within the Atlantic population. Indeed, the analysis of 160 bp showed a significant genetic differentiation between Moroccan coast and adjacent seas.

Key Words: cytochrome b, haplotype diversity, nucleotide diversity, Clade A, Clade B, Alboran Sea, Atlantic.

Résumé. L'anchois Européen (*Engraulis encrasicolus*) est un poisson pélagique qui joue un rôle socio-économique important, régulièrement ciblé par les pêcheurs au Maroc. Dans cette étude, la fréquence des clades et la structure génétique des populations d'anchois européen collectées au niveau de quatre zones géographiques marocaines ont été étudiées en utilisant un fragment de 682 pb du gène du cytochrome b. L'analyse a porté sur un total de 138 individus. Les résultats ont mis en évidence 118 haplotypes et 147 sites de ségrégation (S) dont 59 étaient parcimonieusement informatifs. Une valeur élevée a été également enregistrée pour la diversité des haplotypes $h = 0,987 \pm 0,006$. Par contre, la diversité nucléotidique a été relativement faible avec une valeur de $0,0089 \pm 0,0047$. Nos analyses montre aussi qu'au niveau des côtes marocaines, le clade d'anchois européen dominant est le Clade A avec un pourcentage allant de 89% en Mer d'Alboran à 95% sur la côte atlantique. Dans l'ensemble, il y avait une différenciation génétique significative entre les 4 zones marocaines ($\Phi_{st} = 0,013$; $p = 0,039$). Les Φ_{st} s par paires ont révélé une différenciation génétique entre la population d'Alboran (Méditerranée) et les populations de l'Atlantique, surtout lorsque on n'utilise que les séquences du clade dominant (Clade A), alors que la différenciation génétique a été faible ou modérée au sein des populations de l'Atlantique. En effet, l'analyse de 160 pb a indiqué une différenciation génétique significative entre la côte marocaine et les mers adjacentes.

Mots-clés: cytochrome b, la diversité haplotypique, la diversité nucléotidique, Clade A, Clade B, La mer d'Alboran, l'Atlantique.

Introduction. The European anchovy, *Engraulis encrasicolus* (Linnaeus, 1758) is a small pelagic fish found in a wide range of temperatures (2–30°C) and salinities (5–41 psu) in the eastern Atlantic, the Mediterranean Sea and the Black Sea (Whitehead et al 1988). This species plays a major socio-economic role in all regions; it is one of the principal

target species for commercial fisheries. Indeed, European anchovies with other Engraulidae fisheries representing 14 percent of the world catch of fish (Ababouch & El Marrakchi 2009). In Morocco anchovy fisheries are very important with thousands tons annually cashed (Table 1). So, with the other small pelagic fishes, anchovies play a role in the national economy, they are source of foreign exchange and animal protein, (Belvez 1984). In addition their economic importance, anchovies like other pelagic fish occupy crucial positions in the oceans ecosystems (Ganias 2014) particularly in upwelling regions where it occupies fundamental intermediate trophic level (Bakun 2006). As is known, small pelagic are characterized as 'wasp-waist', being considered as crucial components of pelagic ecosystems (Cury et al 2000). In addition, anchovy as a pelagic species, it can achieve considerable biomass and undergoes crash–flush cycles (Uriarte et al 1996; Lavoué et al 2007). For these reasons, the management of this precious resource is important. In fact, anchovies have been the subject of many genetic studies aimed a better understanding of the stock genetic structure as identifying stocks, discriminating among them, and determining the stock composition of mixed stocks are integral elements of fishery management (Waldman 1999). Likewise, genetics and fishery management can interact in several ways. When the genetic population structure of a species is known, the distribution of subpopulations in mixed fisheries can be estimated (Utter 1991).

Table 1

Commercial product of the coastal and artisanal fishing of *Engraulis encrasicolus* in Morocco (<http://www.onp.ma/statistiques>)

Year	2010	2011	2012	2013	2014	2015
Weight (T)	25,783	28,915	34,983	15,980	9,414	16,695
Value K. USD	16,606	11,109	12,736	8,268	7,733	13,826

T - tons, K. USD - 1 000 US Dollar.

Table Supp. 1

Sample information and Genbank access number of sequences used in this study

	Note	Sequence accession numbers
Morocco	Present study	KT246173-KT246289
	France	EU224051-EU224052
Bay of Biscay	Atlantic Ocean	KF972469-KF972479
	Nantes	JN103357-JN103370
	***	EF427558-EF427559
Cantabric Sea	Spain	FN688316-FN688318
	Lienres coasts	JN103332-JN103356
	Viscaya	JN103386-JN103386
	Mauguio	KF972518-KF972520
Western Mediterranean	Italy	FR851443-FR851450
	***	EF439526-EF439527
	***	KF972521-KF972524
	France	FN688314-FN688315

Previous published data using allozymes, mitochondrial DNA (mtDNA) RFLPs, control region sequence, microsatellites and SNP marker (Bembo et al 1996a,b,c; Tudela et al 1999; Magoulas et al 1996, 2006; Kristoffersen & Magoulas 2008; Sanz et al 2008; Zarraonaindia et al 2009; Borrell et al 2012; Viñas et al 2013; Silva et al 2014;) suggest the presence of various genetically differentiated groups with spatial and ecological components. In addition to this, Magoulas et al (1996, 2006) by analyzing mitochondrial

DNA restriction fragment length polymorphism, reported a significant phylogeographic structure in both the Atlantic and Mediterranean populations of anchovy with two haplotype clades (A and B) separated by 3.2% sequence divergence. Grant (2005), Viñas et al (2013), Oueslati et al (2014) and Silva et al (2014) confirmed the existence of these clades whose frequency varies within samples across the Mediterranean and Northeast Atlantic.

Despite the importance of this species, no widespread genetic studies have focused on the European anchovy population from Moroccan coasts. The objective of this work is to deal with genetic structure of anchovy from this region by using mitochondrial DNA and to examine the hypotheses of existence of two clades (A and B) in the Moroccan coasts as it was reported in other region.

Material and Method

Fish sampling. Fish was carried out using INRH RV "Amir Moulay Abdallah" during species spawning period in 2012, from three Moroccan Atlantic locations, the fourth sample was collected from Moroccan Alboran Sea (Figure 1) (See Field Study Permissions).

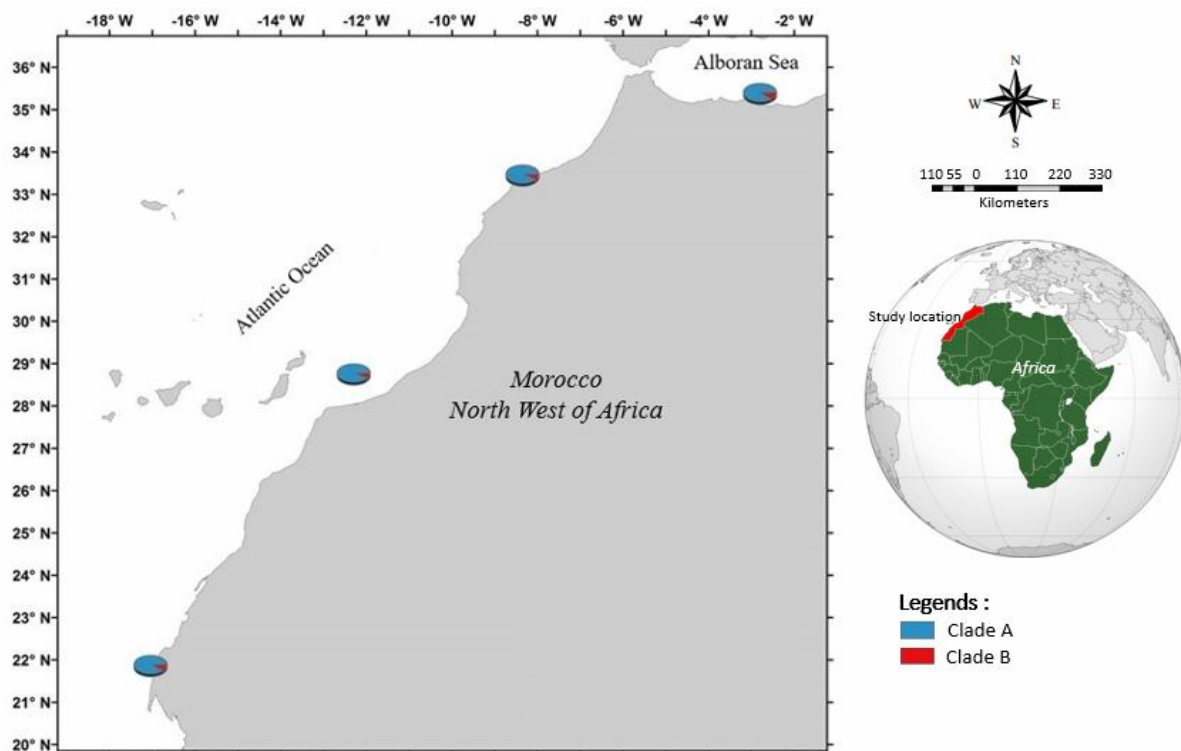


Figure 1. Map of Moroccan Coast showing sampling sites and mitochondrial frequencies.

Indeed, in this kind of study it is strongly recommended to take samples in spawning season, in which adults return to their spawning areas generation after generation, known as reproductive homing (Brochier et al 2009). This behavior, characterized by a migration process coupled with the recognition of natal watershed sites, is rewarded by an increase in reproductive success and will evolve into genetically determined phenotypes (Brochier et al 2009; Lucas & Baras 2001). Also, different population structures and spatial patterns in genetic variability should be expected when examining either juvenile or adult populations (Telles et al 2011).

DNA extraction, amplification and sequencing. DNA was extracted from 25 mg of fin using the "QIAGEN DNeasy Blood & Tissue kit" following the manufacturer recommendations. A total of 138 individuals were used for mtDNA analyses (Table 2 and

Figure 1). For PCR, a fragment of 680 bp was amplified with an initial denaturation at 95°C for 10 min, followed by 35 cycles (95°C 60 s; 52°C 1 min, 72°C 1 min) with a final extension at 72°C for 10 min using the primers 5'-AACGACGCAGTAGTAGACC' and 5'-GAGGAAGTACTCACTCAGGC- defined in positions 42 and 825 of the cyt-b locus of *E. encrasicolus*, respectively. The amplification products were sequenced at the CNRST/Morocco Platform and 680 bp aligned for individuals between positions 108 and 787 of the gene in BIOEDIT (Hall 1999) with ClustalW, using the mtDNA cytochrome b sequence of the *E. encrasicolus* (sequence accession numbers GENBANK KF873783 and KF874179) as a reference of clade with manual adjustments (Oueslati et al 2014). Also, the BIOEDIT was used to align the sequences obtained with those from the GenBank collected from Bay of Biscay, Cantabric Sea and Western Mediterranean (Table 1) between the position 526 and 685 of the gene.

Table 2

List of sample and genetic statistics within each of the sample sites for cytochrome b mitochondrial gene in *Engraulis encrasicolus*

Sample	Sample code	FAO fishery area	Date	N	$h \pm s.d.$	$n \pm s.d.$	Tajima's D
Moroccan Alboran Sea	Albo	37.1.1	Oct-12	37	0.998±0.007	0.0118±0.0017	-1.887* ±0.914
North Atlantic of Morocco	N_ATL	34.1.11	Nov-12	25	0.967±0.029	0.0070±0.0013	-1.977* ±0.894
Central Atlantic of Moroccan	C_ATL	34.1.12 & 34.1.13	Jan-13	44	0.993±0.008	0.0070±0.0008	-2.432* ±0.927
South Atlantic of Moroccan	S_ATL	34.1.3	March-12	32	0.968±0.024	0.0093±0.0018	-2.202* ±0.912
Total	-	-	-	138	0.987±0.006	0.0089±0.0047	-2.124* ±0.244

* significant values for $p < 0.05$; N - sample size; h - haplotype diversity; n - nucleotide diversity.

Table Supp. 2

Pairwise Φ_{st} among our samples and nears waters using 160 bp of cytochrome b mitochondrial gene sequences

	Alboran	ATL_N	ATL_S	ATL_C	B.B	C. Sea	W.M
Alboran	0.00000						
ATL_N	0.00000	0.00000					
ATL_S	0.00311	0.00000	0.00000				
ATL_C	0.02153	0.00508	0.00000	0.00000			
Bay of Biscay	0.09965*	0.13617*	0.10821*	0.19337*	0.00000		
Cantabric Sea	0.15985*	0.19962*	0.16801*	0.26375*	0.00000	0.00000	
Western Mediterranean	0.44560*	0.51542*	0.44257*	0.57798*	0.13590*	0.10272*	0.00000

* significant values after Bonferroni correction.

Data treatment. The phylogenetic relationships among mtDNA cyto b haplotypes were evaluated by neighbour-joining (NJ) analysis using MEGA 6.06 (Tamura et al 2013). DnaSP version 5.10.1 (Librado & Rozas 2009) was used to calculate nucleotide diversity per site (π) (Nei 1987) and haplotype diversity (h) (Nei & Tajima 1981) from haplotype frequencies and haplotype divergence. The Tajima's D (Tajima 1989) was also estimated

to assess the likelihood that the DNA sequences have evolved in a neutral manner with significance tests (1 000 simulations) (significant, $P < 0.05$). Pairwise genetic differentiation between samples (Φ_{st}) was estimated in Arlequin 3.5.3.1 (Excoffier & Lischer 2010) on 10,000 permutations. In addition, to determine homogenous groups of populations, analysis of molecular variance "AMOVA" of haplotypes was carried out in Arlequin 3.5.3.1 with 1,000 permutations to establish significance.

Results and Discussion. Among all the samples, the Clade A was the most common. Thus, 92% had a haplotype from Clade A and 8% had a haplotype from Clade B. The frequency of Clade A was high (89%) in the Alboran Sea, but its prevalence was lower than in the Moroccan Atlantic coast with a percentage that ranged from 91% in south of Moroccan Atlantic to 98% in the Central of Moroccan Atlantic (Figure 2).

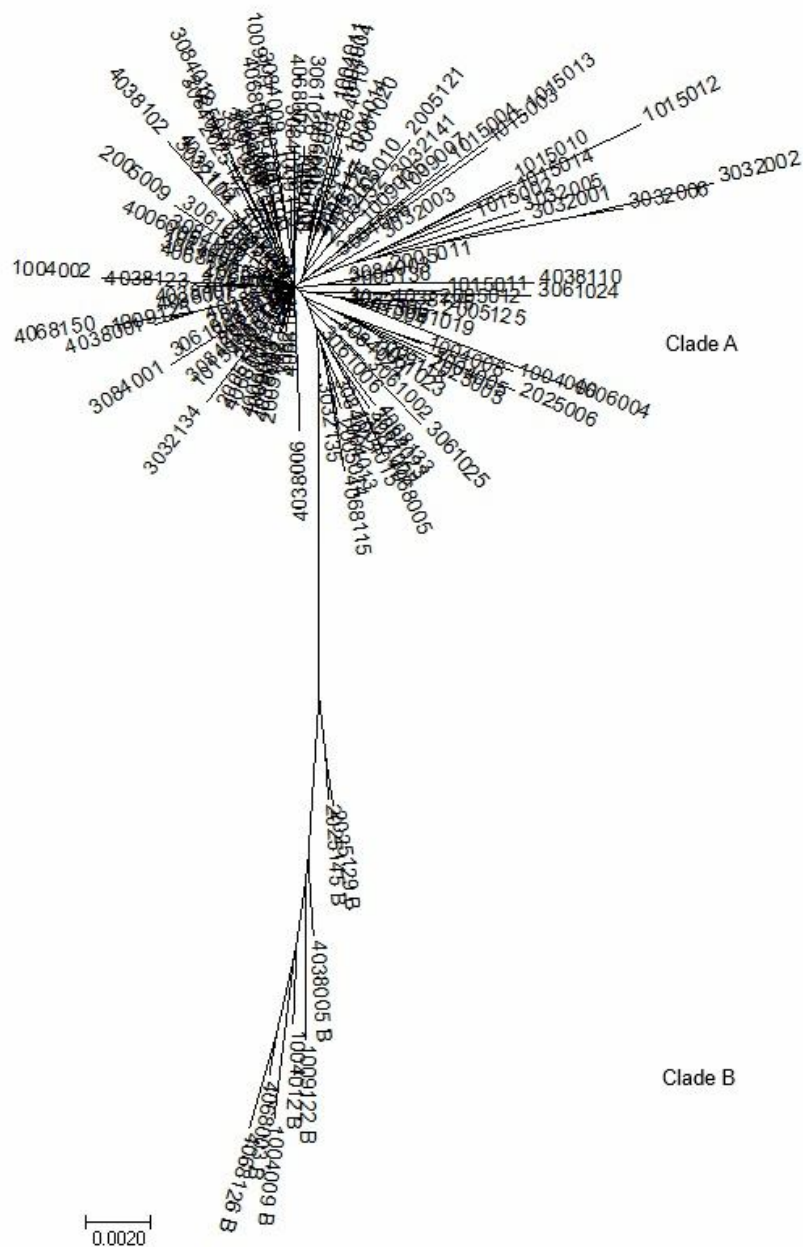


Figure 2. Unrooted NJ tree showing the relationship of mDNA cytochrome b.

The polymorphisms investigation defined 118 distinct *E. encrasicolus* mtDNA haplotypes and 147 segregating sites (S), 59 of which were parsimoniously informative sites. Higher levels of genetic variation were found for the haplotype diversity $h = 0.9868 \pm 0.0061$, but

a concatenated mean nucleotide diversity for all samples was 0.008946 ± 0.004740 (Table 2). The haplotype sequences were deposited in GenBank, with accession number KT246173-KT246289. All locations had a close haplotype diversity, whereas for the nucleotide diversity the Moroccan Atlantic samples were less variable than the Alboran samples, which had the higher nucleotide diversity ($n = 0.01185 \pm 0.00166$).

Genetic structure of *E. encrasicolus* populations was analyzed by pairwise Φ_{st} values and AMOVA. Overall, there was significant genetic differentiation among the 4 populations ($\Phi_{st} = 0.01283$; $p = 0.03519$). Pairwise Φ_{st} revealed a strong homogeneity within Atlantic populations. A largest and significant genetic differentiation occurred between Alboran and Atlantic populations when using only Clade A sequences (Table 3). The AMOVA analysis using only Clade A sequences showed that genetic variations observed were mainly among groups (group 1: Alboran samples and group 2: Atlantic samples); $F_{ct} = 0.03293$, $p < 0.05$. This value decreases when we include all dataset; 0.0253 , $p = 0.00489$. Significant genetic differentiation was observed between Moroccan population and the other three populations (Bay of Biscay, Cantabric Sea and Western Mediterranean), and Pairwise Φ_{st} values ranged from 0.09965 to 0.57798 , suggesting that the Moroccan population was genetically distinct from the other populations (Table 2).

Table 3

Pairwise Φ_{st} among the 4 samples. Below the diagonal using the whole dataset samples and above using only Clade A sequences

Location	Alboran	N_ATL	C_ATL	S_ATL
Alboran	0	0.02004*	0.02764*	0.03181*
N_ATL	0.01015	0	0.00087	0.00069
C_ATL	0.02990*	0.00409	0	0.00277
S_ATL	0.01291	0.00000	0.00702	0

* significant values after Bonferroni correction (Sample codes as in Table 2).

Tajima D tests were performed to test whether the Cyt b fragment evolved under neutrality or not. Under the infinite mutation model, The Tajima D tests resulted in negative and significant values ($P < 0.05$) for each population independently, or for all individuals (Table 2) indicating an excess of low-frequency haplotypes which can arise from selection or rapid population growth in the Moroccan coast (Tajima 1989).

Clade frequency. The two mitochondrial clades (A and B) reported in the previous studies were present in our samples, despite at highly variable frequencies. Thus, the Moroccan coast, that either the Alboran Sea or the Atlantic Ocean, were predominantly harboring by the clade A (about 90 %). Some regional patterns in mtDna frequency have been already acknowledged although seldom scrutinized at the local scale like our work. So, the dominance of clade A in the Alboran and Atlantic in Moroccan coast was similar to near waters like the western Portuguese coast, Bay of Cadiz, Canary Islands, the central African coast and the Alboran Sea (Spanish and Algerian coast) (Magoulas et al 2006; Viñas et al 2013; Oueslati et al 2014; Silva et al 2014), while the frequency of haplotypes from clade A was lower in the northwestern Mediterranean, black Sea, Aegean Sea, Tunisia coast (Tyrrhenian Sea and Central Mediterranean) and Cantabrian Sea. By contrast, the Adriatic Sea, Ionian Sea, Northern North Sea, Baltic Sea and English Channel were characterized by a high frequency of clade B (>82%), with a slight cline in the Bay of Biscay (Magoulas et al 1996; 2006; Borrell et al 2012; Viñas et al 2013; Oueslati et al 2014; Silva et al 2014). Magoulas et al (2006), by using RFLPs in mtDNA, exhibited that the sequence divergence between two clades was 3.2%. The heterogeneity of source between these two matriarchal populations has been attributed to a combination of present day isolation and historical biogeography events (see Magoulas et al [1996, 2006] and Grant [2005] for an appraisal of their phylogeographic history).

Population structure. The marine species appear to have a lower degree of geographic differentiation than continental species. This phenomenon is typically explained by a high dispersal rate due to a planktonic egg and larval phase of many of these species, or adult history stages coupled with an absence of physical barriers to movement, which makes them a priori poor candidates for differentiation at small scales (Grant & Bowen 1998). Despite this, in our study, AMOVA and pairwise Φ_{st} revealed that there is a genetic differentiation among samples of *E. encrasicolus* in the Alboran Sea and Atlantic Ocean in the Morocco ($\Phi_{st} = 0.01283$; $p = 0.03519$). In fact, there is a small signal, which may assume the presence of a population in Alboran Sea genetically different from Atlantic populations, particularly when we use only sequence of dominant clade (Clade A). These results are consistent with other studies conducted on European anchovies sampled from Alboran Sea and Atlantic Ocean. Using RFLPs in mtDNA, Magoulas et al (2006) reveal that the constriction of the Strait of Gibraltar partially isolates the Mediterranean from the eastern North Atlantic. In the same vein, using a multi-marker approach, Zarragonaindia et al (2012) propose the existence of two ecological groups; one major group of Iberian-Atlantic populations was associated with upwelling areas on narrow continental shelves and includes populations spawning and overwintering in coastal areas. A second major group includes northern populations in the North East (NE) Atlantic (including the Bay of Biscay) and the Mediterranean was associated with wide continental shelves with local larval retention currents. Based on the sequence variability in the mitochondrial (mtDNA) control region, Viñas et al (2013) found that the Alboran population was genetically distinct from other Mediterranean and Northeast Atlantic populations, including neighboring populations; Cadiz. Likewise, by microsatellites, Silva et al (2014) provide evidence of the existence of genetic structure between Malaga (in the Alboran Sea) and Portugal south, Tangier, Canaries and Senegal (in the Atlantic Ocean).

Despite the absence of a true physical or hydrological barrier to gene flow between the Alboran Sea and the Atlantic Ocean, one fundamental difference between *E. encrasicolus* and other coastal pelagic species, which show little genetic population subdivision, is that *E. encrasicolus* inhabit complex coastal seas isolated from one another by Peninsulas and narrow straits; as the Strait of Gibraltar (Magoulas et al 2006), and this complexity tends to isolate populations by reducing of gene flow levels between regions. Thus, the constriction of the Strait of Gibraltar could partially isolates the Alboran population from the Atlantic population and this may also explain the mosaic structure of the clade frequency. In addition to physical barriers imposed by complex shorelines, the pelagic ecosystem of the Moroccan Atlantic coast is influenced by the upwelling phenomenon which appears in accordance with Zarragonaindia et al (2012) hypothesis on the presence of two ecotypes; one associated to upwelling areas and the second associated to wide-shelf areas characterized by larval-retention mechanisms. Furthermore, Viñas et al (2013) explained the genetic distinctness of Alboran population might result from the endogenous recovery of the population after the fishery collapse in the 1990s (Irazola et al 1996; Pertierra & Leonart 1996 in Viñas et al 2013) that led to the accumulation of rare alleles, such as those detected in allozymes (Sanz et al 2008).

In the Moroccan Atlantic coast (35.83 °N-21 °N), a homogeneous population was detected, whereas we expected a structuring in parallel 30.63 °N, as in the sardine (*Sardina pilchardus*) (Chlaida et al 2008), because this area is characterized by the presence of a complex hydrodynamic environment. Opposite to *S. pilchardus* which have a wintering spawning period, anchovy spawns mainly in summer (July and August) (Berraho 2007). In this period hydrological barrier, completely onset in winter for *S. pilchardus* (Chlaida et al 2008), is permeable for anchovy in summer because of the shift in the hydrodynamic environment. Johnson & Stevens (2000) and Stevens & Johnson (2003), explained that there is a difference in vertical circulation depending on the season in the Canary Current Upwelling System induces changes in physical factors which would become favorable to mixture between genitors and larval dispersal during anchovy spawning period (Chlaida et al 2008; Chahdi Ouazzani et al 2016). Besides, a higher temporal scale, the history of *E. encrasicolus* could also explain the low genetic differentiation observed in the Atlantic. The results of Tajima test indicate that the population of the Atlantic has probably been subjected to recent population expansion.

Indeed, a high level of haplotype diversity and low nucleotide diversity of mtDNA may be the result of rapid population growth from an ancestral population of small sizes, provided the time was sufficient for recovery of haplotype variation via mutation yet too short for an accumulation of large sequence differences (Avice 2000).

The interaction of the Atlantic jet with the denser and more saline Mediterranean water results in the formation of a quasi-continuous geostrophic front along the northern edge of the gyres and at the eastern boundary of the Eastern Alboran Gyre (EAG), which is known as the Almeria-Oran Front (AOF). As suggested in numerous studies (Bembo et al 1996b; Magoulas et al 2006; Bouchenak-Khelladi et al 2008; Sanz et al 2008), as well as in many other species (Patarnello et al 2007), the AOF is a dispersal barrier which separates the population of anchovies from either side of the front; Alboran and Western Mediterranean. The AOF northern end detaches from the Spanish coast between Almeria and Cartagena, while its southern end terminates around Oran on the North African coast (Sanchez-Vidal et al 2004) and breaking the gene flow between Atlantico-Alboran population and the Western Mediterranean population. In our study we showed that the Clade A was dominant along the Moroccan coast, Whether Alboran Sea or the Atlantic Ocean, while the haplotypes clade A frequency was lower in Cantabrian Sea and the Bay of Biscay (see above), which explains that the western Moroccan Atlantic areas may be genetically distinct from the Bay of Biscay and Cantabrian Sea.

Conclusions. In this work we confirm the dominance of Clade A on the Moroccan coast and we detect a difference between the Alboran Sea and the Atlantic, although more suitable sampling methods and more sensitive genetic markers is needed to confirm our results. We believe that additional genetic data can provide insights that will improve the sustainable management of *E. encrasicolus* Moroccan fisheries.

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Field study permissions. Sampling and analysis techniques used in this work are consistent with the Moroccan Legislation Article 14, Section 3, Bill 122.22 and the Cartagena Protocol on Biosafety to the Convention on Biological Diversity promulgated by the royal decree "1.09.123".

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