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Genetic polymorphism and population structure analysis of blue mussels (*Mytilus* spp.) in the north-western Mediterranean coasts of Morocco

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Abstract

Genetic polymorphism and population structure of blue mussels (*Mytilus* spp.) from five locations (Cap Spartel, Belyounesh, M'diq, Martil and Azla) in the north-western coasts of Morocco were analyzed using partial DNA sequences from the F and M- types mitochondrial control regions. All genetic diversity parameters revealed higher variability in the M than in the F type. The negative values of neutrality tests suggest that the five studied populations could be in a recent expansion process. Moreover, genetic differentiation statistics supported by AMOVA analyses demonstrated that mussel populations from the north-western coasts of Morocco are genetically undifferentiated.

Keywords: mtDNA polymorphism, genetic differentiation, AMOVA

Introduction

The blue mussel *Mytilus galloprovincialis* Lmk is distributed in Europe from the Mediterranean Sea to the English Channel and the British Isles (Lubet *et al.*, 1984; Garner, 1994; Seed, 1992). Moreover, *M. galloprovincialis* coexists and hybridises with *Mytilus edulis* L in some areas of the Atlantic French and British coasts (Coustau *et al.*, 1991; Hoeh *et al.*, 1991; Sanjuan *et al.*, 1994). *M. galloprovincialis* is generally considered to be genetically homogeneous in a particular geographic area, although substantial genetic differentiation has been reported for the southeastern Iberian populations (Quesada *et al.*, 1995a; Sanjuan *et al.*, 1994). This genetic break corresponds to a discontinuity in the distribution of this mussel that is associated with the Almeria-Oran oceanographic front, which acts as a distributional boundary between Atlantic and Mediterranean communities (Quesada *et al.*, 1995a; Sanjuan *et al.*, 1994).

Mussel species of the genus *Mytilus* carry two types of mitochondrial DNA (mtDNA) genomes, one that is transmitted maternally (F type) and another that is transmitted paternally (M type) (Skibinski *et al.*, 1994; Breton *et al.*, 2006). Normally, females are homoplasmic for an F type whereas males are heteroplasmic for a maternal F and a paternal M type (Skibinski *et al.*, 1994; Zouros *et al.*, 1994, Cao *et al.*, 2004, Cao *et al.*, 2009).

Determination of the mussel species that inhabit Atlantic and Mediterranean Moroccan coasts has been controversial. In this sense, earlier studies based on morphological markers reported that *M. edulis* as well as *M. galloprovincialis* were present on both Atlantic and Mediterranean coasts of Morocco (Bellon-Humbert, 1973; Lubet, 1973; Zine & Menioui, 1992). However, Daguin & Borsa (1999), using a single DNA marker, suggested that north-western African *Mytilus* were *M. galloprovincialis*. Recently, allozyme markers revealed the exclusive existence of *M. galloprovincialis* on Mediterranean and Atlantic coasts of Morocco (Jaziri & Benazzou, 2002). Interestingly, it has been showed that a significant multilocus discontinuity in allozyme markers separate southern Atlantic populations from Mediterranean and north Atlantic ones, due to a sea surface current direction change from Cap Ghir towards the Canaries archipelago (Jaziri & Benazzou, 2002). Nevertheless, little information about the level of genetic population structuring and polymorphism in north-western Moroccan mussel populations based on mtDNA sequences is available.

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In this study, we have investigated the mtDNA polymorphism and genetic differentiation among populations of the genus *Mytilus* from five locations of the north-western coasts of Morocco: Cap Spartel, Belyounech, M'diq, Martil, and Azla. For this purpose, we have analyzed partial sequences of the rapidly evolving mitochondrial control region in both F and M type genomes. Genetic and statistical analyses for both F and M haplotypes were conducted.

Materials & Methods

Mussel samples used in the present work were collected during November 2004 in five locations from the north-western Moroccan coasts (Fig. 1): Cap Spartel (35º47´N; 5º56´W), Belyounech (35º55´N; 5º24´W), M'diq (35º41´N; 5º19´W), Martil (35º37´N; 5º16´W) and Azla (35º33´N; 5º14´W). A total of 24 individuals were sampled from each location.

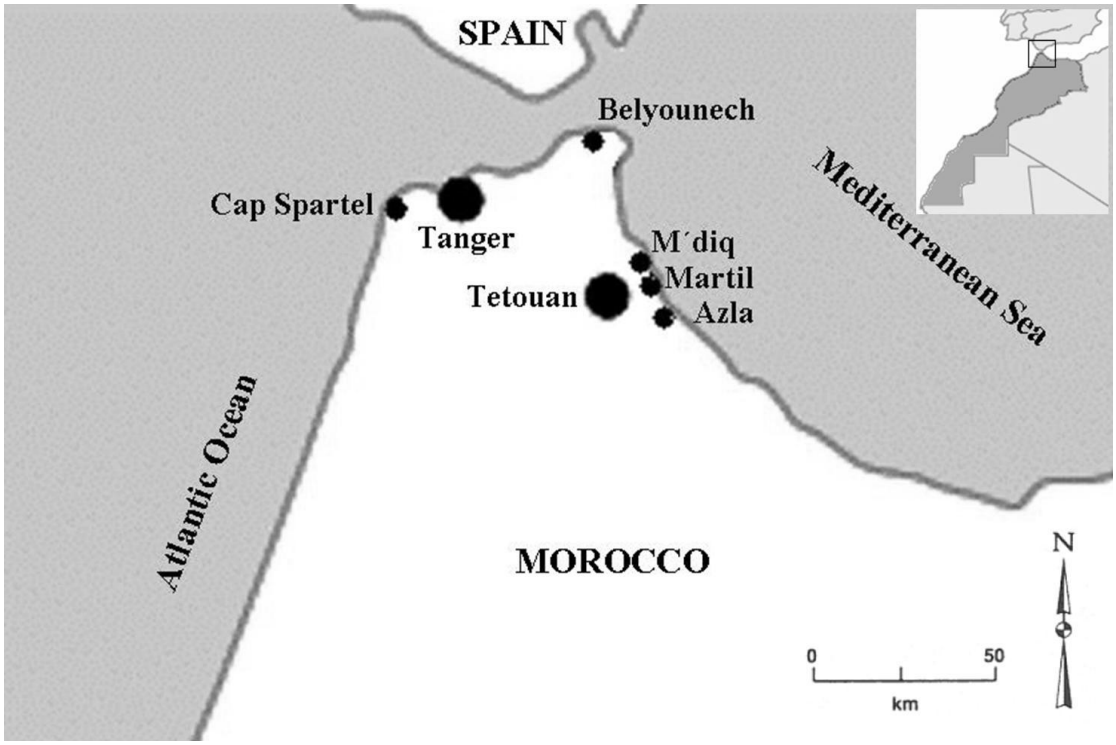

Figure 1: Map of the north-western coasts of Morocco showing the sampling sites of *Mytilus* spp.: Cap Spartel, Belyounech, M´diq, Martil, and Azla.

Total genomic DNA was isolated from 150 mg of each tissue using FastDNA® kit during 40 s and speed setting 5 in the Fastprep® FG120 instrument (Bio101, Inc). All DNA isolation procedure was performed following the manufacturer's protocol.

For PCR amplification of a partial fragment of F and M type mitochondrial control region, three primers were designed using the software Oligo® v6.82 (Medprobe) based on sequences retrieved from GenBank/EMBL/DDBJ databases corresponding to *M. edulis* (F: AF315573; M: AF188279) and *M. galloprovincialis* (F: AF188278; M: AF188280) control region. The most conserved areas were localized to assure an efficient amplification. For F type, the forward primer MytRC•1 (5'-TTGGAATAGATGCAGGAGATGGGGGCTTA-3') and the reverse primer MytRC•2 (5'- TTTCAAACCCAGGTAAATCTCGTGAGCAACAG-3') were used. For the M type, the same reverse primer MytRC•2 was used together with the forward primer MytRC•3 (5'-AGGTGTTTCTACACGCTTAGATTCCTTGCCATT-3'). Reactions were carried out in 25 µl of reaction volume: 1 µl of DNA template (~30-50 ng) was

added to 24 µl of PCR mix consisting of 17.25 µl of sterile distilled water, 2.5 µl of dNTP mix 10 mM, 2.5 µl of 10x buffer, 1 µl of $MgCl_2$ 50 mM, 0.25 µl (1.25 units) of BioTaq™ DNA polymerase (Bioline, London, UK) and 0.5 µl of each primer (10 µM). The thermal cycle profile was identical for all the amplified fragments. An initial denaturation step of 96 ºC for 2 min was followed by 35 cycles of 96 ºC for 30 s, 60 ºC for 30 s, and 72 ºC for 1 min. PCR products were electrophoresed on a 2 % agarose gel and visualized via ultraviolet trans-illumination before sequencing.

Double-stranded DNA products were purified using a PCR product purification kit (Marlingen Bioscience) and subsequently sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on a 3130 Genetic Analyzer (Applied Biosystems). The obtained DNA sequences were analyzed using the computer programs Sequencing Analysis version 3.4.1 (Applied Biosystems) and Seqman v5.51 (DNASTAR).

Both F and M sequences were aligned using Megalign v5.51 software (DNASTAR). Haplotype diversity (Hd, Nei, 1987), nucleotide diversity (π, Nei, 1987), and other DNA polymorphism parameters were estimated using DNAsp *v*4.0 (Rozas *et al.*, 2003). The same program was used to calculate several tests of neutrality such as Fu and Li's D and F (Fu & Li, 1993) and Tajima's D (Tajima, 1989) statistics.

Population genetic differentiation was assessed by mean of different statistical tests based on DNA sequences: K_{ST}, K_S and Z (Hudson *et al.*, 1992), and S_{nn} (Hudson, 2000). The genetic structure within and among populations was also analyzed using Wright's F_{ST} statistic (Wright, 1978) from the values of haplotype frequencies and Tamura-Nei genetic distances between DNA sequences (Tamura & Nei, 1993). The global value of F_{ST} statistic was also calculated from analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992). Permutation procedures were used to test the significance of the different statistical parameters. For multiple tests, we used the sequential Bonferroni adjustment as advocated by Rice (1989). Partitioning of genetic variability among populations was tested using AMOVA (Excoffier *et al.*, 1992) with ARLEQUIN *v*2.0 software (Schneider *et al.*, 2000).

Results

Twenty-four specimens from five locations of the north-western coasts of Morocco were analyzed. Amplicons of variable sizes were obtained for both F and M mtDNA types. A total of 56 and 40 different haplotypes were identified among 113 F and 46 M type sequences, respectively.

	Cap Spartel	Belyounech	M'diq	Martil	Azla
Individuals	22	22	24	23	22
Number of haplotypes	16	14	16	16	17
Haplotype diversity (Hd)	0.97	0.92	0.93	0.96	0.97
Average number of differences (K)	4.892	3.900	5.772	4.486	5.364
Number of polymorphic sites (S)	31	21	37	26	26
Nucleotide diversity (π)	0.007	0.006	0.009	0.007	0.008
Nucleotide polymorphism (θ)	0.013	0.009	0.017	0.012	0.013

Table 1: Genetic diversity parameters for F type genome in five natural populations of *Mytilus* spp. from the north-western coasts of Morocco.

Genetic variation of each geographic population was estimated from the analysis of three different statistics: Haplotype diversity (Hd), number of polymorphic sites (S), average number of pairwise nucleotide differences, nucleotide diversity (π) and nucleotide

polymorphism (θ). Estimated values for the F type genome are depicted in Table 1. Belyounech revealed as the most homogeneous population exhibiting the lowest values for all parameters. In contrast, M'diq showed the highest statistic values except for Hd that reached the maximum (0.97) in Azla and Cap Spartel.

	Cap Spartel	Belyounech	M'diq	Martil	Azla
Individuals	8	6	6	13	13
Number of haplotypes	8	6	6	12	13
Haplotype diversity (Hd)	1	1	1	0.99	1
Average number of differences (K)	4.821	6.000	18.733	3.295	5.295
Number of polymorphic sites (S)	13	12	36	14	22
Nucleotide diversity (π)	0.016	0.012	0.037	0.008	0.012
Nucleotide polymorphism (Φ)	0.014	0.015	0.047	0.014	0.022

Table 2: Genetic diversity parameters for M type genome in five natural populations of *Mytilus* spp. from the north-western coasts of Morocco.

Table 2 depicts the statistic values for the M type genome. All five populations showed high Hd values (0.99-1.00). The population of Martil appeared to be the most homogeneous, whereas K, S, π and θ parameters reached their maximum values in M'diq.

	F type genome			M type genome		
Populations	Fu and Li's D	Fu and Li's F	Tajima's D	Fu and Li's D	Fu and Li's F	Tajima's D
Cap Spartel	–3.030**	–3.073*	–1.716	–1.416	–1.517	–1.204
Belyounech	–2.647*	–2.624*	–1.323	–0.966	–1.034	–0.913
M'diq	–2.400	–2.586	–1.775	–1.295	–1.421	–1.312
Martil	–2.465	–2.539	–1.505	–2.015	–2.226	–1.767
Azla	–1.333	–1.526	–1.248	–2.187	–2.427*	–1.947*

Table 3: Results of the Fu and Li's D and F and Tajima's D statistical tests of neutrality.

Pairwise comparisons	K_S	K_{ST}	*P*	Z	*P*	S_{nn}	*P*	$F_{ST(HF)}$	*P*	$F_{ST(TN)}$	*P*
CS vs. BL	4.40	0.00	0.828	475.32	0.563	0.54	0.193	0.014	0.169	0.000	0.824
CS vs. MD	5.35	0.00	0.754	521.25	0.764	0.48	0.590	0.001	0.379	0.000	0.742
CS vs. MA	4.68	0.00	0.863	499.48	0.821	0.54	0.233	0.000	0.687	0.000	0.861
CS vs. AZ	5.13	0.00	0.775	476.72	0.766	0.47	0.595	0.000	0.862	0.000	0.575
BL vs. MD	4.88	0.00	0.266	516.21	0.327	0.49	0.475	0.218	0.070	0.000	0.451
BL vs. MA	4.20	0.00	0.931	499.83	0.897	0.42	0.901	0.000	0.811	0.000	0.981
BL vs. AZ	4.63	0.00	0.425	473.85	0.477	0.50	0.447	0.007	0.278	0.000	0.378
MD vs. MA	4.14	0.00	0.584	541.68	0.485	0.54	0.181	0.006	0.267	0.000	0.589
MD vs. AZ	4.58	0.00	0.636	521.42	0.789	0.45	0.742	0.000	0.615	0.000	0.609
MA vs. AZ	4.92	0.00	0.517	496.87	0.558	0.57	0.122	0.000	0.620	0.000	0.501

CS: Cap Spartel, BL: Belyounesh, MD: M'diq, MA: Martil, AZ: Azla.

Table 4: Analyses of genetic differentiation among Moroccan mussel populations based on F type genome. Pairwise comparisons for F_{ST} statistic were carried using haplotype frequencies ($F_{ST(HF)}$) and Tamura-Nei distances ($F_{ST(TN)}$).

The null hypothesis of neutrality in the mutation process was evaluated using Fu and Li's D and F (Fu & Li, 1993) and Tajima's D (Tajima, 1989) neutrality tests. As it is shown in Table 3 for F and M genome, negative values were obtained for these statistics in all populations, although they were significant only in some cases.

No genetic differentiation among populations was observed for F type genome using three sequence-based statistics (K_{ST}, Z, and S_{nn}) (Table 4). For the M type genome, significant values were obtained only in some Cap Spartel *vs* Azla pairwise comparison tests (Table 5); however, these values were not significant after application of sequential Bonferroni corrections (Rice, 1989).

Pairwise comparisons	K_S	K_{ST}	*P*	Z	*P*	S_{nn}	*P*	$F_{ST(HF)}$	*P*	$F_{ST(TN)}$	*P*
CS vs. BL	4.10	0.000	0.515	45.14	0.482	0.59	0.589	0.000	0.999	0.000	0.436
CS vs. MD	7.70	0.003	0.349	45.00	0.445	0.60	0.601	0.000	0.999	0.039	0.148
CS vs. MA	3.30	0.004	0.348	102.85	0.230	0.56	0.272	0.000	0.516	0.016	0.262
CS vs. AZ	4.09	0.028	0.032	100.53	0.048	0.58	0.252	0.000	0.999	0.054	0.039
BL vs. MD	8.50	0.008	0.261	32.28	0.331	0.47	0.447	0.000	0.999	0.015	0.245
BL vs. MA	3.45	0.003	0.310	83.51	0.228	0.47	0.774	0.000	0.999	0.031	0.170
BL vs. AZ	4.32	0.009	0.205	84.80	0.358	0.41	0.886	0.000	0.999	0.024	0.196
MD vs. MA	5.90	0.007	0.181	83.48	0.215	0.60	0.264	0.000	0.999	0.092	0.083
MD vs. AZ	6.87	0.009	0.115	83.98	0.256	0.46	0.763	0.000	0.999	0.080	0.059
MA vs. AZ	3.62	0.000	0.842	162.00	0.453	0.59	0.918	0.000	0.999	0.000	0.807

CS: Cap Spartel, BL: Belyounesh, MD: M'diq, MA: Martil, AZ: Azla.

Table 5: Analyses of genetic differentiation among Moroccan mussel populations based on M type genome. Pairwise comparisons for F_{ST} statistic were carried using haplotype frequencies ($F_{ST(HF)}$) and Tamura-Nei distances ($F_{ST(TN)}$).

The level of genetic differentiation was also evaluated by using F_{ST} statistic based on haplotype frequencies and Tamura-Nei genetic distances (Excoffier *et al.*, 1992). Similarly to K_{ST}, Z, and S_{nn} statistics, no significant differences were detected among populations for the F (Table 4) and M (Table 5) type genomes. As a whole, these results indicate absence of genetic differentiation among the populations analyzed.

Source of variation	d.f.	Sum of squares	Variance components	Pourcentage of variation
Using haplotype frequencies				
Among populations	4	1.940	0.00047 Va	0.10
Within populations	108	51.228	0.47433 Vb	99.90
Total	112	53.168	0.47481	
Using Tamura-Nei distances				
Among populations	4	6.953	–0.03058 Va	–1.27
Within populations	108	262.354	2.42921 Vb	101.27
Total	112	269.308	2.39863	

Table 6: Hierarchical analysis of variance under the AMOVA framework for F type genome.

To assess the level of genetic structuring among populations, we estimated the partitioning of genetic variability among and within populations using AMOVA (Excoffier *et al.*, 1992). This analysis can be carried out using either data of haplotype frequencies within each population or values of genetic distances between sequences. However, when high levels of Hd are detected, AMOVA based on of genetic distances between sequences is the most

appropriate. In our case, Tamura-Nei distance was applied to correct for multiple hits in order to account for transition/transversion rate and base-composition biases, as well as substitution rate variations among different sites (Tamura & Nei, 1993). The results of AMOVA corresponding to F type genome are shown in Table 6. Most of the total genetic variability was found within populations whatever the method used, while the among-populations variance was very low. The F_{ST} value associated to AMOVA was zero in both cases. Similar results were obtained for the M type genome (Table 7). In this case, among-populations variance was not detected using haplotype frequencies, and it was smaller than 4% with the genetic distances method. F_{ST} values associated to AMOVA were zero for haplotype frequencies, and 0.03 for genetic distances. Therefore, it can be concluded that the five studied populations are genetically undifferentiated.

Source of variation	d.f.	Sum of squares	Variance components	Pourcentage of variation
Using haplotype frequencies				
Among populations	4	1.816	−0.00494 Va	−1.00
Within populations	41	20.423	4.49812 Vb	101.00
Total	45	22.239	0.49318	
Using Tamura-Nei distances				
Among populations	4	13.945	0.09977 Va	3.70
Within populations	41	106.430	2.59586 Vb	96.30
Total	45	120.375	2.69563	

Table 7: Hierarchical analysis of variance under the AMOVA framework for M type genome.

Discussion

Genetic variation of each geographic population was estimated from the analysis of different parameters: i) Hd, that takes values ranging from 0 when all haplotypes are similar, and 1 with unrepeated haplotypes (Nei, 1987), ii) the number of polymorphic sites (S), iii) the average number of pairwise nucleotide differences (K; Tajima, 1983), iv) nucleotide diversity (π) assessed as the average number of nucleotide differences between all possible pairs of sequences, with values ranging from 0 when no variation is detected and 1 in the case of extreme divergence among haplotypes (Nei, 1987), and v) nucleotide polymorphism (θ), defined as the proportion of polymorphic sites in the analysed sequences (Nei, 1987).

Previous studies with the aim to determine the level of genetic differentiation among mussel populations in Moroccan coasts are found in the literature. Thus, Daguin & Borsa (1999), analyzing the actin gene locus *mac-1*, revealed that mussel population from Tetouan (35º04'N, 5º04'W) in the Mediterranean coast did not exhibit genetic differences with the Atlantic population from Temara near Rabat (33º55'N, 6º54'W). The same authors demonstrated that such populations were more similar to others from the north-eastern Atlantic than from the Mediterranean Sea. This abrupt genetic transition between Atlantic and Mediterranean *M. galloprovincialis* mussels was also signaled in others studies based on allozymes (Quesada *et al.*, 1995a), nuclear markers (Daguin *et al.*, 2001), or mitochondrial DNA (Quesada *et al.*, 1995b; Sanjuan *et al.*, 1996). Such transition is associated to the Almeria-Oran oceanographic current. In fact, The Alboran Sea is actually considered to be a permanent intrusion of Atlantic surface waters in the Mediterranean, with the Almeria-Oran oceanographic front delimiting it at the East (Tintore *et al.*, 1988; La Violette, 1989) and constituting the effective boundary between the Atlantic and the western Mediterranean waters. This physical structure may constitute a barrier to larval flow, thus explaining the abrupt genetic change between Atlantic and Mediterranean mussels. Nevertheless, Jaziri & Benazzou

(2002), analyzing allozyme markers of mussel populations along all Moroccan coasts, revealed that a significant multilocus discontinuity separate southern Atlantic populations (at the south of Essaouira, 31º30'N, 9º45'W) from Mediterranean and north Atlantic (from Essaouira to Gibraltar strait) ones. This transition could be explained by a gene flow breaking because of a larval dispersal decrease, due to a sea surface current direction change from Cap Ghir (30º37'N, 9º54'W) towards the Canaries archipelago (Mittelstaedt, 1991). Our results based on sequence analysis of partial mtDNA control region highlight the absence of any genetic differentiation among *M. galloprovinciallis* from the north Atlantic-Mediterranean region, which is in accordance with the previously cited results based on allozyme markers. In conclusion, additional analyses of the mtDNA control region of mussel populations from Atlantic coasts of Morocco will allow elucidating if the allozyme genetic discontinuity is accompanied by a mtDNA transition.

The higher polymorphisms detected in M type in relation to F type genome could be a consequence of the apparently negligible role of the M type in somatic tissues, with absence of selective pressure (Hoeh *et al.*, 1996). With regard to this, our results are in agreement with those obtained by different authors studying another mtDNA regions (Rawson & Hilbish, 1995; Skibinski *et al.*, 1994; Stewart *et al.*, 1995; Smietanka *et al.*, 2009).

Fu and Li's D neutrality test is based on the differences between the number of singletons (mutations appearing only once among the sequences) and the total number of mutations, whereas Fu and Li's F test is based on the differences between the number of singletons and the average number of nucleotide differences between pairs of sequences within each population. By the other side, Tajima's D statistic is based on the difference between two estimates of the amount of variation. One estimate is obtained from the number of segregating sites (Watterson, 1975) and the other is based on π (Nei, 1987; Tajima, 1983). In a constant-size neutral equilibrium population, the expectation of Tajima's D is nearly zero because the expectations of both estimates are the same. Negative values obtained in this work for all these statistics would indicate that the studied populations could be in a recent expansion process after experiencing founder effect or population bottleneck.

The sequence-based statistics (K_{ST}, Z, and S_{nn}) uses the information on the number of nucleotide differences between haplotype sequences, and are more appropriate methods when the Hd are very high and the sample sizes are small (Hudson *et al.*, 1992). The K_{ST} statistic was calculated as $K_{ST} = 1 - (K_S/K_T)$, where K_S is a weighted average of K_1 and K_2 (the average number of differences between sequences from within populations 1 and 2, respectively) and K_T is the average number of differences between two sequences regardless of their population. Under the null hypothesis of no differentiation, we expect K_{ST} to be near zero, and so we will reject the null hypothesis (P<0.05) if K_{ST} is too big. Similarly, a small value of K_S would lead to rejection of the null hypothesis (P<0.05). Another statistic, Z, referred to as "rank statistic", was also evaluated. It is a weighted sum of Z_1 and Z_2, where Z_i is the average of the ranks of all the $d_{ij.lk}$ values for pairs of sequences from within population *i* (Hudson *et al.*, 1992). The null hypothesis of no differentiation is rejected if the Z statistic is too small (P<0.05). The last statistic was S_{nn}, referred to as the nearest-neighbor statistic, a measure of how often the nearest neighbors of sequences are found in the same population (Hudson, 2000). If a population is strongly structured, one expects to find the nearest neighbor of a sequence in the same population. Thus, for two populations of the same size, S_{nn} is expected to be near 1 when the populations are highly differentiated and near 0.5 when the two populations are part of the same panmictic population (P<0.05). In our case, the whole results indicated that no genetic differentiation was detected among the analyzed populations.

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الملخص العربى

التباين الوراثي وتحليل تركيب الجماعات فى بلح البحر الأزرق (*Mytilus* spp) فى السواحل الشمالية الغربية للبحر المتوسط فى المغرب

عوفى سمير [1] ، مانويل مانشادو [2] ، كارلوس أنفاتى[2] ، أوجينيا زواستى [2] ، أنيلا كريسبو [2] ، يونس سعود [1]
1. قسم البيولوجى – كلية العلوم – تيتوان – المغرب
2. مركز تورينو- تيرو دى بيشون – بورتو يى سانتا ماريا - أسبانيا

تم تحليل اختلاف الأشكال الوراثية وتركيب جماعات بلح البحر الأزرق (*Mytilus* spp.) الموجودة في خمسة مواقع على السواحل الشمالية الغربية للبحر المتوسط فى المغرب (كاب سبارتل وبليونش والمضيق ومرتيل وأزلا) وذلك باستخدام التسلسل الجزئي للحمض النووي المأخوذ من نوعى مناطق التحكم الـ F و الـ M للميتوكوندريا. وكشفت عوامل التنوع الجيني كلها عن مقدار عالي من التباين في النوع الـ M عن النوع الـ F. وتشير القيم السالبة لاختبارات التعادل إلى أن الجماعات الخمسة قيد الدراسة يمكن أن تكون خاضعة لعملية توسع حديثة. وعلاوة على ذلك ، أظهرت التحليلات الإحصائية باستخدام تحليل الأنوفا، فيما يخص التمييز الجيني أن جماعات بلح البحر الموجودة على سواحل شمال غرب المغرب غير مميزة جينياً.