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Phylogenetic and introgression analyses of mitochondrial DNA in five natural populations of mussels (*Mytilus* spp.) from the North-Eastern Coasts of Morocco

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Abstract
Mussel species of the genus *Mytilus* have two types of mitochondrial DNA (mtDNA), the M type, transmitted from male parents to their sons, and the F type, transmitted maternally. In the present survey, we have determined the level of polymorphism of both mtDNA types in mussels from five locations sampled along the north-eastern Moroccan coasts. PCR technique was used to amplify 113 F type and 46 M type partial sequences of the mtDNA control region. A total of 56 and 40 different haplotypes from F and M mtDNA types were identified, respectively. Phylogenetic analyses revealed that both F and M lineages were closely related to *Mytilus edulis*. Five individuals from each of the analyzed populations were randomly selected, and assayed for the nuclear Glu-5'. A DNA band pattern compatible with *Mytilus galloprovincialis* was identified in all cases. These findings lead to the conclusion that a high level of introgression of *M. edulis* mtDNA into *M. galloprovincialis* occurs in this geographical area.

Key words: mitochondrial DNA, phylogenetic relationships, Glu-5' marker, introgression, *Mytilus galloprovincialis*, *Mytilus edulis*

Introduction

The blue mussel *Mytilus galloprovincialis* (Lamarck, 1819) distributes in the Atlantic from the southern coast of England and Ireland to the Iberian peninsula, and throughout the Mediterranean and the Black Sea (Lubet *et al*. 1984, Gardner 1992, Seed 1992). The close phylogenetically related *Mytilus edulis* (Linnaeus, 1758), by constrast, has a more boreal distribution in the western Atlantic Ocean (Garrido-Ramos *et al*., 1998). Hybrid zones along the western European coasts between both species (west France, west Ireland, southwest England) have been reported, showing a mosaic structure with populations of parental genotypes alternating with mixed populations (Skibinski *et al*. 1983, Coustau *et al*. 1991, Gardner 1994, Bierne *et al*. 2003). On the contrary, concordance between mophological variation and enzyme polymorphisms indicates that only *M. galloprovincialis* occurs all along the Iberian Peninsula (Sanjuan *et al*., 1994). Similarly, the exclusive presence of *M. galloprovincialis* has been reported along the Moroccan coasts based on allozyme markers (Comesaña *et al*. 1998, Jaziri & Benazzou 2002) and intron-length polymorphism at the actin gene locus *mac-1* (Daguin & Borsa, 1999), although no mtDNA characterization of Moroccan mussel populations has been carried out to date.

Marine mussels of the genus *Mytilus* carry two types of mitochondrial genomes, one that is transmitted maternally to offspring of both sexes, named F type, and another that is transmitted paternally to male progeny only, named M type (Skibinski *et al*. 1994, Zouros *et al*. 1994). This unique mechanism of transmission of two gender associated mitochondrial genomes (Skibinski *et al*. 1994b, Zouros *et*

al. 1994b) has been called doubly uniparental inheritance (DUI). In the DUI system females are homoplasmic for an F genome, while males are heteroplasmic for a maternal F and a paternal M genome, although this heteroplasmy is unconventional in the sense that somatic tissues are dominated by the F genome and the gonad by the M genome (Garrido-Ramos *et al*., 1998).

Hybridization between *M. edulis* and *M. galloprovincialis* is common in natural mussel populations along the coasts of western Europe (Skibinski *et al*. 1983, Coustau *et al*. 1991, Gardner 1994, Daguin *et al*. 2001, Hilbish *et al*. 2002, Bierne *et al*. 2003). As a result, several surveys have shown the introgression of *M. edulis*-like mtDNA into *M. galloprovincialis*. For example, mitochondrial haplotypes corresponding to Mediterranean *M. galloprovincialis* were generally absent in Atlantic *M. galloprovincialis*, being detected in only a putative hybrid population in southwest Britain (Rawson *et al*. 1999, Hilbish *et al*. 2000). In a similar way, phylogenetic analyses of paternal and maternal mtDNA haplotypes grouped in two separate clusters, one containing Mediterranean *M. galloprovincialis* (rare in Atlantic *M. galloprovincialis*), and the other one containing all haplotypes found exclusively in *M. edulis*, together with haplotypes found in both Atlantic and Mediterranean *M. galloprovincialis* (Quesada *et al*., 1998). The fact that most Atlantic and a proportion of Mediterranean *M. galloprovincialis* carry *M. edulis*-like haplotypes indicates they have been introgressed by *M. edulis*-like mtDNA.

The mussel polyphenolic adhesive protein is a key component in the attachment of mussels to the substratum, and is encoded by the *Glu* locus (Rawson *et al*., 1996). The primary sequence of this protein is highly divergent between *M. edulis* and *M. galloprovincialis* (Filpula *et al*. 1990, Inoue & Odo 1994), which has allowed the design of a specific and direct PCR-based assay (referred to as Glu-5' marker) for the identification of *Mytilus* species based on nuclear DNA (Rawson *et al*., 1996). The aim of the present work was to analyze the existence of mtDNA introgression in mussels of the genus *Mytilus* from five locations of the north-eastern coasts of Morocco: Cap Spartel, Belyounech, M'diq, Martil, and Azla. For this purpose, we have analyzed the rapidly evolving mitochondrial control region (Aquadro & Greenberg 1983, Desjardins & Morais 1990, L'Abbe *et al*. 1991) in both F and M type genomes. Phylogenetic analyses for both F and M haplotypes were conducted. Additionally, the nuclear Glu-5' marker was analyzed for species identification.

Material and methods

Sampling

Mussel samples used in the present work were collected during November 2004 in five locations from the north-eastern Moroccan coasts (Figure 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.

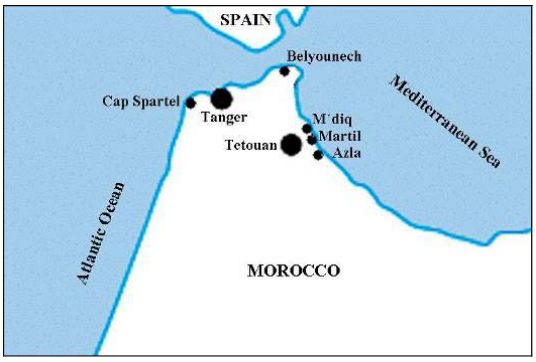

Figure 1. Sampling sites for *Mytilus* spp.: Cap Spartel, Belyounech, M´diq, Martil and Azla.

DNA isolation

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.

PCR amplification, purification and sequencing

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 database 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'-TTGGAATAGATGCAGGAGATGGGGG CTTA-3') and the reverse primer MytRC•2 (5'-TTTCAAACCCAGGTAAATCTCGTGAG CAACAG-3') were used. For the M type, the same reverse primer MytRC•2 was used together with the forward primer MytRC•3 (5'-AGGTGTTTCTACACGCTTAGATTCCT TGCCATT-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).

Sequence and phylogenetic analyses

Both F and M sequences were aligned using Megalign v5.51 software (DNASTAR). DnaSP v4.10.3 (Rozas *et al*., 2003) was used to estimate the number of polymorphic sites among F and M sequences, as well as the frequency of each haplotype.

For the stablishment of phylogenetic relationships among F haplotypes, reference sequences retrieved from the GenBank/EMBL/DDBJ database corresponding to *M. edulis* (Accession No. AF315573) and *M. galloprovincialis* (Accession No. AF188278) control region were also employed. Similarly, for M types, reference sequences for *M. edulis* (Accession No. AF188279) and *M. galloprovincialis* (Accession No. AF188280) control region were added. In each case, Modeltest v3.06 software (Posada & Crandall, 1998) was employed as a guide to determine the best-fit maximum likelihood (ML) model as previously described (Cunningham *et al*., 1998). Neighbor-joining phylogenetic analysis of both F and M haplotypes was implemented in PAUP*4.0b10 (Swofford, 2000) using the ML distance settings. The degree of confidence assigned to nodes in trees was achieved by bootstrapping (Felsenstein, 1985) with 1000 iterations. In addition, a minimum spanning network was built from the matrix of absolute numbers of substitution differences between all pairs of haplotypes using the program TCS v1.13 (Clement *et al*., 2000). The network was drawn by hand based on the output of the programme.

Analysis of Glu-5' marker

Reactions were conducted in a final volume of 12.5 µl containing 50 ng of total DNA, 2.5 nmol of dNTPs, 50 pmol of each of the primers JH-5 and JH-54 (Rawson *et al*., 1996), 1.5 mM of $MgCl_2$, 1x buffer, and

0.5 U of BioTaq™ DNA polymerase. The thermal cycle profile was as follows: an initial denaturation step of 3 min at 94 ºC was followed by 30 cycles of 94 ºC for 20 s, 53 ºC for 20 s, and 72 ºC for 45 s. PCR products were then examined by electrophoresis on ethidium bromide-stained 2.5% agarose gel and visualized via ultraviolet trans-illumination. A 100 bp DNA ladder (Invitrogen) was used as molecular weight standard.

A **B**

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                          1111111111 1111222222 2333333334 4444444444 4555555555 55566666
     12223345 6677788899 0001222355 7889113889 9001456770 0013445667 8034456688 88900011    Haplotype frequency (%)
    3800370227 3514926936 1498348216 5051022046 8679286231 4713475179 1974831413 48912902
H01 CTG-CG-TCT TCACTATGCC CTTGCTGAGC CCCCGTGCAC CTGGCTGCTC CCCATCAAAC GCGACAATGA TA-GGGAG          1.77
H02 ...-..-.T. ..G.CT.... ....T..... T.T.....A. .C..T..... ......AA.. ........A. ..-....A         15.93
H03 ...-..-... ..G.CT.... ....T..... T.T.....AT .C..T..... ......AA.. ........A. ..-....A          2.65
H04 ...-..-... .......... ....T..... T.......A. ....T..... ......AA.. .T......A. .G-.....          0.88
H05 ...-.A-.T. ..G.CT.... ....T..... T.T.....A. .C..T..... ......AA.. ........A. ..-....A          0.88
H06 ...-TT-... ....CT.... ....T..... T.TT....A. .C..T..... .....TAA.. ........A. ..-....A          0.88
H07 ...-TC-... ....CT.... ....T..... T.T.....A. .C..T..... T.....AA.. ........A. ..-....A          2.65
H08 ...-..-... ..G.CT.... ....T..... T.T.....A. .C..T..... ......AA.. ........A. ..-....A          3.54
H09 ...-TC-C.. ....CT..T. ....T..... T.T.....A. .C..T..... ......AA.. ........A. ..-....A          0.88
H10 ...-TC-... ....CT.... ....T..... T.T.....A. .C..T....T ......AA.. ........A. ..-....A          0.88
H11 ...-..-.T. ..G.CT.... ....T..... T.T....TA. .C..T..... ......AA.. ........A. ..-....A          0.88
H12 ...-TC-... ....CT.... ....T..... T.TT....A. .C..T..... ......AA.. ........A. ..-....A          0.88
H13 ...-TC-... ....CT.... ....T..... T.T.....A. .C..T..... ......AA.. ........A. ..-....A         13.27
H14 ...-TC-... ....CT.... ....T..... T.T.....A. .C..T..... .....GAA.. ........A. ..-....A          0.88
H15 ...-..-.T. ..G.CT.... ....T..... T.T.....A. .C..T..... ......AA.. .......AA. ..-....A          0.88
H16 ...-..-.T. ..G.CT.... ....T..... T.T.....A. .C..T..... ......AA.. ......C.A. ..-....A          2.65
H17 ...-TC-... ....CT.... ....T.A... T.T.....A. .C..T..... ......AA.. ........A. ..-....A          0.88
H18 ...-..-.T. ..G.CT.... ....T..... T.T.....A. .C..T..... ......AA.. ........AT ..-....A          0.88
H19 ...-..-... .......... ....T..... T.......A. ....T..... ......AA.. ........A. .G-..A..          0.88
H20 ...-.C-... ....CT.... ....T..... T.T.....A. .C..T..... ......AA.. ........A. ..-....A          1.77
H21 ...-TC-C.. ....CT.... ....T..... T.T.....A. .C..T..... ......AA.. ........A. ..-....A          1.77
H22 ...A..-.T. ..G.CT.... ....T..... T.T.....A. .C..T..... ......AA.. ........A. C.-....A          0.88
H23 ...-TC-... ....CT.... ....T..... T.T.....A. .C..T..... ....C.AA.. ........A. ..-....A          6.19
H24 ...-TC-... ..G.CT.... ....T..... T.T.....A. .C..T..... ......AAT. ........A. ..-....A          0.88
H25 ...-..-.T. ..G.CT.... ....T..... T.T.....A. .C..T..... ......AA.T ........A. ..-....A          0.88
H26 ...-TC-... ....CT.... ..C.T..... T.T.....A. .C..T..... ......AA.. ........A. ..-....A          3.54
H27 ...-.C-... ..G.CT.... ....T..... T.T.....-. .C..T..... ......AA.. ........A. ..-AA..A          0.88
H28 ...-..-..A ..G.....T. ....T..... T.T.....A. TC..T..... ......AA.. ........A. ..-.....          1.77
H29 ...-..T.T. ..G.CT.A.. ....T..... T.T....TA. .C..T..... ......AA.. ........A. C.-....A          0.88
H30 ...-TC-... ....CT.... .......... T.T.....A. .C..T..... ......AA.. ........A. ..-....A          1.77
H31 ...-TC-... ....CT.... ....T..... T.T.....A. .C.AT..... ....C.AA.. ........A. ..-....A          0.88
H32 ...-TC-... ....CT.... ....T..C.. T.T.....A. .C..T..... ......AA.. ........A. ..-....A          0.88
H33 ...-TC-... ....CT.... ....T..... T.T.....A. .C..TC.... T.....AA.. ........A. ..-....A          0.88
H34 ...-..-.T. ..G.CT.... ....T..... TTT..C..A. .C..T..... ......AA.. ........A. ..-....A          1.77
H35 ..A-..-.T. ..G.CT.... ....T..... T.T.....A. .C..T..... ......AA.. ........A. ..-....A          0.88
H36 ...-..-.T. ..G.CTC... ....T..... T.T.....A. .C..T..... ......AA.. ........A. ..-....A          0.88
H37 ...-..-.T. ..G.CT.... ...AT..... T.T.....A. .C..T..... ..T...AA.. ........A. ..-....A          0.88
H38 ...-TC-... ....CT.... ....T..... T.TT....A. .C..T..... .....T-A.. ........A. ..-....A          0.88
H39 ...-TC-... ....CT.... ....T..... T.T.....A. .C..T..T.. ......AA.. ........A. ..-....A          0.88
H40 ...-TC-... ....CT.... ....T..... T.TT....A. .C..T..... .....TAA.. ........A. ..-....A          1.77
H41 ...-.A-.T. ..G.CT.... T...T..... T.T.....A. .C..T..... ......AA.. ........A. ..-....A          0.88
H42 .G.-..-... .TG..... ..G.T...AT T.......A. .C..T..... .T....AA.. A...G...A. ..A...G-          0.88
H43 ...-..-... ..G.CT.... ....T..... T.T.....A. .CA.T..... ......AA.. ........A. ..-....A          0.88
H44 ...-..-..A ..G.....TT ....T..... T.T....TA. TC..T.A... ...T..AA.. .....C..A. ..-....A          0.88
H45 ...-..-.T. ..GTCT.... ....T..... T.T.....A. .C..T..... ......AA.. ........A. ..-....A          0.88
H46 ...-..-.T. ..G.CT.... ....T..... T.T.....A. .C..T..... .T....AA.. ........A. ..-....A          0.88
H47 ...-..-.T. ..G.CT.... ....T..... T.T.....A. .C..T..... T.T...AA.. .......... ..-....A          0.88
H48 ...-TC-C.. ....CT...T ....T..... T.T.....A. .C..T..... ......AA.. ........A. ..-....A          0.88
H49 ...-TC-C.. ....CT.... TCC.TC.... T.T.....A. .C..T..... ......AA.. ........A. ..-....A          0.88
H50 ...-..-... ..G.CT..G. ....T..... T.T.A.A.A. .C..T..... ......AA.. ...T....A. ..-....A          0.88
H51 ...-..-.T. ..G.CT.... ....T..... T.T.....A. .C..T..... ......AA.. .T......A. ..-....A          0.88
H52 ...-TC-... ....CT.... ....T..... T.T.....A. .C..T..... ......AA.. ........A. C.-....A          1.77
H53 ...-..-.T. ..G.CT.... ....T..... T.T.....A. .C..T..... ......AA.. ..T.....A. ..-....A          0.88
H54 G..-TC-... ....CT.... ....T..... T.T.....A. .C..T...C. ......AA.. ........A. ..-....A          0.88
H55 ...-.C-... C...C..... ..C.T..... T.......A. ....T..... ......AA.. ........A. ..-....A          0.88
H56 ...-..-.T. ..G.CT.... ....T..... T.T.....A. .C..T..... ......A-.. ........A. ..-....A          0.88
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Figure 2. A) List of all 78 polymorphic sites (gaps included) found in F type mitochondrial control region haplotypes. Numbers above sites indicate arbitrary positions with respect to the first sequence (H01) in the 635 bp alignment. Dots indicate identity; dashes represent indels. B) Frequency (in percentage of individuals) of each of the 56 different haplotypes.

Results and discussion

Sequence variation

Twenty-four samples from five locations of the north-eastern coasts of Morocco were analyzed. Amplicons of variable sizes were obtained for both F and M mtDNA types. Lenghts were determined to range between 631-633 bp and 350-363 bp in the F type and M type, respectively. A total of 56 and 40 different haplotypes were identified among 113 F and 46 M

type sequences, respectively. In the case of F type, 40 haplotypes were singletons (Figure 2). On the contrary, haplotypes H02 and H13 revealed as the most abundant, being present in 18 (15.93%) and 15 (13.27%) individuals, respectively. For M type, 37 out of 40 haplotypes were singletons, whereas haplotypes H01, H13 and H24 were detected in 2 (4.35%), 5 (10.87%) and 2 (4.35%) individuals, respectively (Figure 3).

A **B**

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                      1 1111111111 1111111111 1111111112 2222222222 2222333333 333333333
     111222233 3334445680 0011111222 3335677778 8888888990 0124445666 7789000111 111133345   Haplotype frequency (%)
    3238058934 5781367341 5801368128 4569307890 1456789053 5112565269 4790347012 345635820
H01 GTATGTGACT ATTCGAACCC ACAAAGGCGT TCCTCGAAAT AAAGCTGCCT ACAAA-AG-T GCTTTGATTG TAAATGGTC          4.35
H02 .......... ........A. ....-..-.. ....T..... .........C ..G..-..-. .......... ......T..      2.17
H03 .......... .......... .......... .......... .........C ......T-. .......... .........      2.17
H04 .......... .......... .......... .......... .......A.C .....-..-. .......... .........      2.17
H05 .......... ........TG .......... .....A.... .........C .....--.-. .......... .........      2.17
H06 ..G.....T. .........G .......... .......... .........C .....-..-. ..AA...... ......A..      2.17
H07 .......... .......... .......... C......... .........C .....-..-. .......... .......C.      2.17
H08 .......... ....A..... .......... .......... .........C ......T-. .......... ......T..      2.17
H09 ......T... ..C....... .......... ........-. .........C .....A..-. .......... .........      2.17
H10 .......... .......... ...T...... .......... .........C .....-..-. .......... .........      2.17
H11 .......... .......... .T........ .......... .........C .T...-..-. .......... .........      2.17
H12 .......... .......... ..G.-..T.. ....T..... .........C .....-..-. .......... ......T..      2.17
H13 .......... .......... .......... .......... .........C .....-..-. .......... .........     10.87
H14 .......... .C......T. .......... .T........ .......G.. .....-..-- .......C.. ......A..      2.17
H15 .......... ........T. ......T... C......... .........C .....--.-. .......... .........      2.17
H16 C......... .......... .......... ........-. .......... .....-..-. .......... .........      2.17
H17 .......... .......... ...T...... ..A....... .........C .....-..-. .......... .........      2.17
H18 .......... .......... .......... ........-. ..-....... .....-..-. .......... .........      2.17
H19 .......... .......... .......... C....A.... .......... .....-..-. .....A.... .........      2.17
H20 ..G....... .........G .......... ........-. .......... .....--.-. .......... .........      2.17
H21 .......... .C........ .......... ........-. .......... ...G.-..-. .......... .........      2.17
H22 .......... .......... .......... ........-. .......... .....-..-. .......... .........      2.17
H23 .......... .......... .......... ...C....-. .......... .....--.-. ....C..... .........      2.17
H24 ....A..... .........G .......... .......... .........C .....-..-. .......... ......A..      4.35
H25 .......... .........G .......... .......... .........C .....-..-. .......... .........      2.17
H26 ......A... .......... .......... .......... .........C .....-..-. .......... .........      2.17
H27 .......... .......... .......... .A........ ........GC .....-..-. .......... .........      2.17
H28 .......... .........G .......... .T......-. .......... .....-..-. .......... .........      2.17
H29 .......... .....C.... .......... .......... .........C .....-..-. .......... .........      2.17
H30 .CC.AC.G.C GC.TA.TTTG GT..TA..AC .A....G--- -G.----T.C G...TA..A. CG...AT--- ----CA...      2.17
H31 .......... G......... .......... .......... .........C .....-..-. .......... ......A..      2.17
H32 .......... .......... .......... .......... .......... .....-..-. .......... ......A..      2.17
H33 .......... ...A...... .......... .....A.... .....C...C .....-..-. .......... ......A..      2.17
H34 .......... .......... .....C.... ........-. .......... .....-..-. .......... .........      2.17
H35 .......... .........G .......... .......... .........C .T...-..-. .......... .........      2.17
H36 .......... .......... .......... .......... .........C .....-..-. .....A.... ........T      2.17
H37 .......... .......... ....-..... ....T..... .........C .....-..-. .......... ......T..      2.17
H38 ...C...... .......... .......... .......... .........C .....-..-. .......... .........      2.17
H39 .......... .........G .......... C......... .........C .....-..-. .......... .........      2.17
H40 .......... .........T .......... .......... .........C .....-..-. .......... .........      2.17
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Figure 3. A) List of all 79 polymorphic sites (gaps included) found in M type mitochondrial control region haplotypes. Numbers above sites indicate arbitrary positions with respect to the first sequence (H01) in the 365 bp alignment. Dots indicate identity; dashes represent indels. B) Frequency (in percentage of individuals) of each of the 40 different haplotypes.

A total of 78 out of 635 sites revealed as polymorphic in the mtDNA F type (Figure 2). These polymorphisms included 113 transitions (41 A↔G and 72 C↔T), and 22 transversions (3 A↔C, 9 A↔T, 3 T↔G, and 7 C↔G). In addition, 7 indels were detected. For the M type, 79 out of 365 sites revealed as polymorphic, including 84 transitions (38 A↔G and 46 C↔T), and 43 transversions (13 A↔C, 10 A↔T, 5 T↔G, and 15 C↔G), plus 22 positions exhibiting indels (Figure 3). The higher polymorphisms detected in M type in relation to F type 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).

Phylogenetic relationships among F and M haplotypes

Modeltest analysis determined the TrN+G model as being the best-fit model of DNA evolution for the F type control region with the following ML parameters: base frequencies were 0.2921, 0.1603, and 0.2584; R(a)=1.0000, R(b)=4.2993, R(c)=1.0000, R(d)=1.0000, and R(e)=15.5565; gamma distribution shape

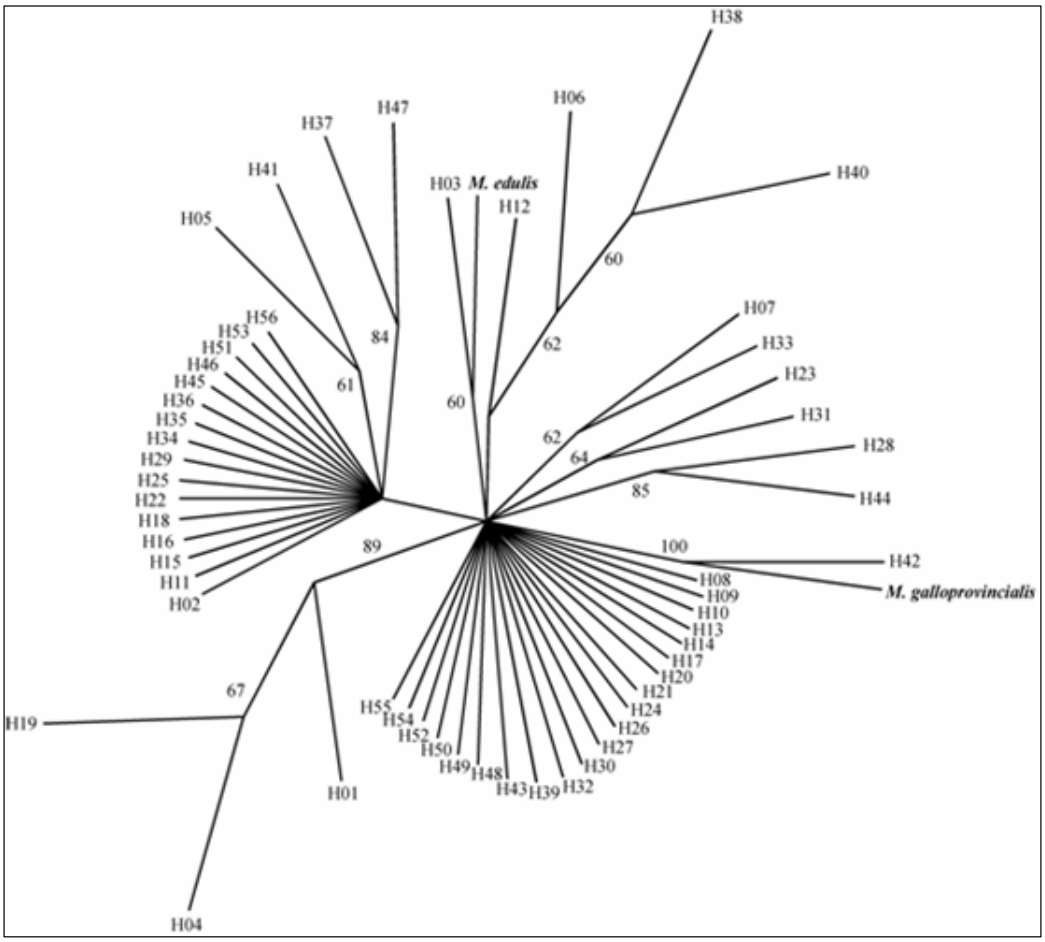

Figure 4. Unrooted neighbor-joining tree showing the phylogenetic relationships of mitochondrial F type haplotypes. Haplotypes are numbered as in figure 2. Only bootstrap values higher than 50% are indicated on the tree.

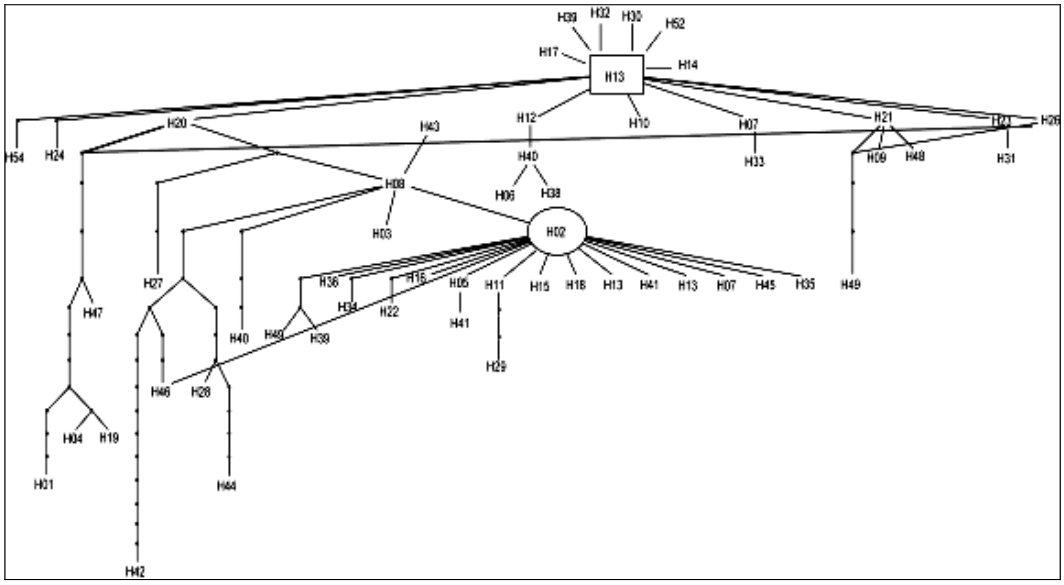

Figure 5. Minimum spanning network of mitochondrial F type haplotypes from *Mytilus* spp. See text for details.

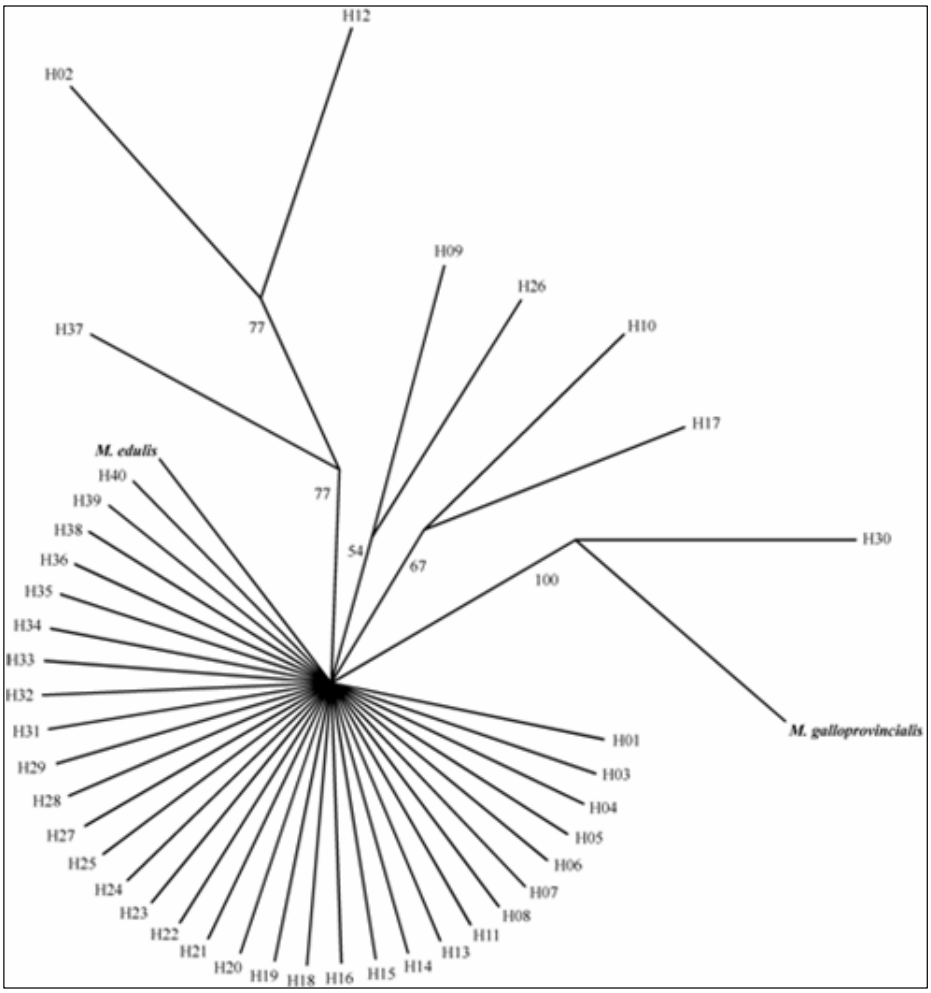

Figure 6. Unrooted neighbor-joining tree showing the phylogenetic relationships of mitochondrial M type haplotypes. Haplotypes are numbered as in figure 3. Only bootstrap values higher than 50% are indicated on the tree.

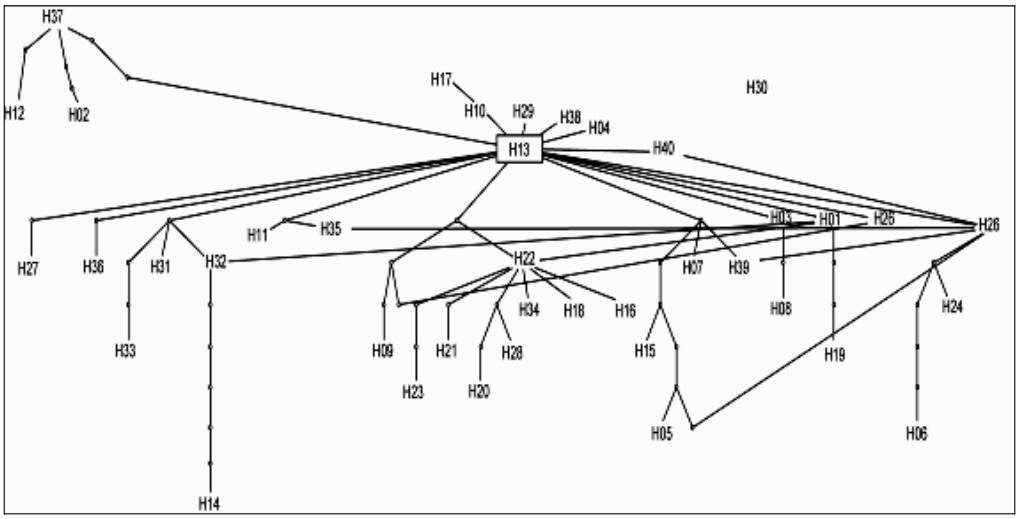

Figure 7. Minimum spanning network of mitochondrial M type haplotypes from *Mytilus* spp. See text for details.

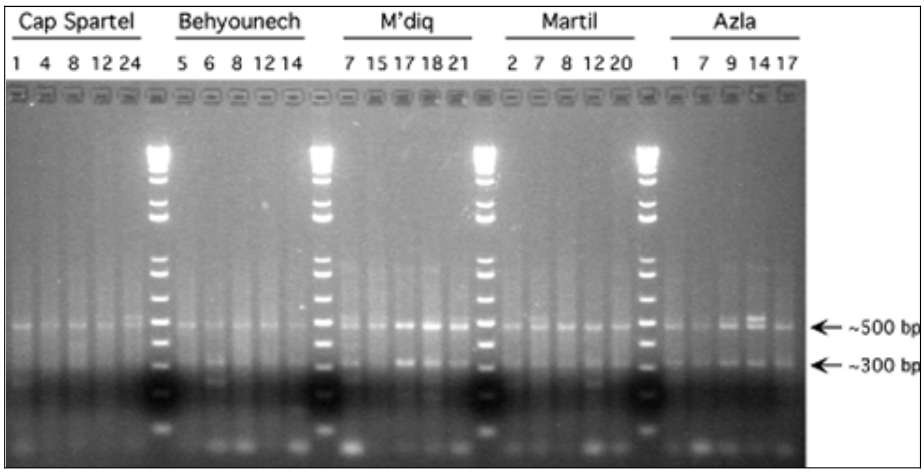

Figure 8. PCR products generated using the Glu-5' assay in five randomly selected individuals of each mussel population. Sizes of the *M. galloprovincialis*-specific PCR products are shown on the right.

parameter was 0.2012. Phylogenetic relationships among F haplotypes were then established using the neighbor-joining method with the maximum likelihood distance settings. The unrooted tree revealed that only haplotype H42 appeared closely linked to *M. galloprovincialis* reference sequence (bootstrap support of 100%), while the other 55 haplotypes were more related to *M. edulis* than to *M. galloprovincialis* (Figure 4).

Relationships among *Mytilus* F haplotypes were also analyzed using a minimum spanning network (Excoffier & Smouse, 1994). The two most abundant haplotypes, H02 (15.93% of individuals) and H13 (13.27%), displayed a central and a star-like position in the mutation network. Thus they should be considered as the most ancestral haplotypes (Figure 5). Most haplotypes were closely related to H02 and H13 with the exception of H42. These results are in agreement with phylogenetic analysis previously described.

In the case of M type haplotypes, the HKY+G model of sequence evolution was the most appropriate as selected by Modeltest with the following parameters: base frequencies were 0.3552, 0.1637, and 0.1886; *Ti/tv* = 3.0047; gamma distribution shape parameter was 0.2148. Phylogenetic relationships were also established using the neighbor-joining method with the ML distance settings (Figure 6). Among the 40 different haplotypes, 39 corresponded to *M. edulis* M type, while only haplotype H30 showed a close relatedness to *M. galloprovincialis*. In fact, this branch exhibited the highest bootstrap support (100%).

Mutational network of M haplotypes had different features in comparison with the previously described for F haplotypes (Figure 7). Haplotype H13, the most abundant one (10.87% of individuals), was observed in a central position connected to the remaining haplotypes by a variable number of mutational changes. The haplotype H30, corresponding to *M. galloprovincialis*, could not be connected to the network due to the excessive differences with respect to the rest of haplotypes. Again, results from mutational network and phylogenetic analysis were in accordance.

Introgression levels as determined by *Glu* locus

Previous surveys based on allozyme differentiation between both species (Comesaña *et al*. 1998, Jaziri & Benazzou 2002) and on intron-length polymorphism at the actin gene locus *mac-1* (Daguin & Borsa, 1999), indicated the exclusive

existence of *M. galloprovincialis* along the Mediterranean and Atlantic coasts of Morocco. Taking into account the high proportion of *M. edulis*-like haplotypes (both F and M) among the samples analyzed, we considered the need to assay the possible occurrence of mtDNA introgression from *M. edulis* into *M. galloprovincialis* largely described in the literature (McDonald & Koehn 1988, Coustau *et al*. 1991, Väinölä & Hvilsom 1991, Comesaña *et al*. 1999, Gardner 1994, Quesada *et al*. 1995, Rawson & Hilbish 1995, Rawson *et al*. 1999, Toro*et al*. 2004).

We performed a molecular analysis in order to detect polymorphisms at the nuclear locus encoding the mussel polyphenolic adhesive protein (*Glu* locus). In the present work, experimental conditions described in Rawson *et al*. (1996) were applied for the Glu-5' marker. In the Glu-5' PCR assay, species-specific banding patterns are produced. In *M. edulis*, a single 350 bp band, and occasionally and additional 380 bp band, is expected. On the contrary, in *M. galloprovincialis* two primary bands of 300 and 500 bp are produced, although some individuals can exhibit only the 500 bp band.

From each one of the analyzed populations, 5 individuals were randomly selected and their Glu-5' marker was amplified by PCR. The results are shown in figure 8. A primary band of 500 pb was identified in all samples, and an additional 300 bp band could be detected in all individuals from Martil and Azla, four individuals from M'diq, and at least one or two individuals in the other two populations. Therefore, nuclear DNA analysis allowed the identification of these mussels as *M. galloprovincialis*, demonstrating the ocurrence of high levels of mtDNA introgression from *M. edulis* into *M. galloprovincialis* in mussel populations along the north-eastern Moroccan coasts. Further research including populations of the west coast will be necessary to assess the overall mtDNA introgression status of *Mytilus* mussels in Morocco.

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