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Genetic characterization of *Mytilus galloprovincialis* Lmk. in North West Africa using nuclear DNA markers

[*MYTILUS GALLOPROVINCIALIS* IN NW AFRICA]

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Abstract

The genetic relationships among *Mytilus galloprovincialis* populations over their range in the northeastern Atlantic and the western Mediterranean were investigated using polymerase chain reaction (PCR)-amplified nuclear DNA markers. We used long-range polyacrylamide gel electrophoresis for characterizing an intron-length polymorphism at the actin gene locus *mac-1* in *Mytilus*. Sharp resolution was obtained with this technique, which revealed a high level of size polymorphism. It also allowed to discriminate between *M. galloprovincialis* and *M. edulis*. *Mytilus* populations sampled on the northwestern African coasts (Morocco, Western Sahara, and Mauritania) were *M. galloprovincialis* as formerly suggested on the basis of morphology and geographic location. A sample of the Padstow mussel reported as *M. galloprovincialis* according to allozyme and morphological data exhibited allele frequencies that were rather intermediate between *M. galloprovincialis* and *M. edulis*. Slight heterozygote deficiencies were possibly present within each sample as usually reported in bivalve populations. Additional PCRs using a second pair of primers showed that this could not be explained by the occurrence of null alleles that would have resulted from mis-priming during DNA amplification. Significant differentiation was observed between *M. galloprovincialis* from northwestern Africa and the reference *M. galloprovincialis* sample from the Mediterranean Sea, a result that is consistent with previous allozyme- and mitochondrial DNA-based reports of an abrupt genetic change between northeastern Atlantic / Alboran Sea and western Mediterranean *M. galloprovincialis* populations.

Key words: mussel; *Mytilus galloprovincialis; Mytilus edulis;* northwestern Africa; polymerase chain reaction; intron; size polymorphism; geographic structure

1. Introduction

The mussel *Mytilus galloprovincialis* Lmk. is present in the northeastern Atlantic from the shores of the Irish Sea to southern Spain and in the western Mediterranean, the Tyrrenian Sea, the Adriatic Sea, the Aegean Sea and the Black Sea (Lubet *et al.*, 1984; Karakousis and Skibinski, 1992; Seed, 1992). Lubet *et al.* (1984) have also reported the presence of *Mytilus* that is morphologically *M. galloprovincialis* in northern Africa. The closely related blue mussel, *M. edulis* L., has a more boreal range. *M. galloprovincialis* and *M. edulis* occur sympatrically from the Irish Sea to the Biscay coast of France or even northern Spain (Lubet *et al.*, 1984; Coustau *et al.*, 1991; Seed, 1992; Gardner, 1994; Sanjuan *et al.*, 1994). *Mytilus* populations in this area exhibit a range of patterns of morphological and genetic intergradation (Skibinski and Beardmore, 1979; Beaumont *et al.*, 1989; Coustau *et al.*, 1991; Koehn, 1991; McDonald *et al.*, 1991; Gardner, 1994) that has been interpreted as the result of extensive hybridization between the two species.

Here we conducted a genetic analysis of *Mytilus* samples from northwest Africa using the intron-length polymorphism at the actin gene locus *mac-1* (Ohresser *et al.*, 1997) as a marker. These samples were compared to reference *M. galloprovincialis* and *M. edulis* samples from, respectively, the western Mediterranean and Denmark (Coustau *et al.*, 1991; McDonald *et al.*, 1991; Seed, 1992) and to a sample of the 'Padstow' (*M. galloprovincialis*-like) mussel from Polzeath Bay in the Irish Sea (Skibinski *et al.*, 1983; Beaumont *et al.*, 1989; Seed, 1992, Ohresser *et al.*, 1997) in order to assess their genetic relationships to other mussel populations in the area. We report that the northwest African *Mytilus* populations, which occur as far south as southern Western Sahara and even Mauritania, are *M. galloprovincialis*, but these also exhibited genetic differences with western Mediterranean *M. galloprovincialis*.

2. Materials and methods

Samples of *Mytilus* spp. were collected in 1995 and 1996 in Denmark, South-West Britain, southern France, Morocco and Western Sahara (details

given in Fig. 1). The sample from Denmark is *M. edulis* according to data from former allozyme studies on samples collected in the area (Coustau *et al.*, 1991; Seed, 1992). Similarly, the sample from southern France has been assigned to species *M. galloprovincialis* (Coustau *et al.*, 1991). We used these two samples as references for either species in subsequent comparisons with the other samples. After their collection, the mussels were either preserved alive on ice (samples from SW Britain and southern France), or frozen (samples from Morocco and Western Sahara) or in alcohol (sample from Denmark) and sent to Laboratoire Génome et Populations in Sète until they were processed as described in the following. Two additional individuals identified as *M. galloprovincialis* on the basis of their morphology were collected amidst dense *Perna picta* Born settlements in Cansado, Mauritania (Fig. 1). These were preserved in 90% ethanol until processed.

For DNA extraction, 1-3 mg adductor muscle tissue was dissected from each individual and digested at 55°C overnight in a microfuge tube containing 6% Chelex (Biorad, Richmond CA, USA) and 12 *U* proteinase K in 350 µl 0.1 m*M* Tris / 0.01 m*M* EDTA, pH 8.0 buffer. The samples were then vortexed, heated at 95°C for 20 min and centrifuged at 3500 *g* for 5 min. The supernatant was stored at -20°C until it was used as the DNA extract for polymerase chain reaction (PCR). Specific PCR primers (forward 5'-CGT CTA GCG TAG TAC TTA AAT TG-3' and reverse 5'-CGA AAA TTG TAG TCT AGT TTT GTG-3') were designed from the alignment of 6 partial sequences of Intron-1 of the actin gene *mac-1* (Ohresser *et al.*, 1997; M. Ohresser, unpublished). The length-polymorphism reported at *mac-1* Intron 1 is due to large (28-65 base pairs (bp)) insertions / deletions and short gaps in the sequences (Ohresser *et al.*, 1997; M. Ohresser, unpublished). We chose the primers that would minimize the length of the PCR product for better electrophoretic resolution while still encompassing most polymorphic sites that were encountered on the 6 sequences. From the information available then we expected that the sizes of the amplified fragments would range from ~250 bp to ~315 bp, a range of sizes that is suitable for separating alleles differing by as little as 1 bp in size when using long-range denaturing polyacrylamide gel electrophoresis.

Prior to PCR the forward primer was radioactively 5' end-labeled using 1 *U*/µl Phage T4 polynucleotide kinase (Eurogentec, Liège, Belgium)

in a reaction mixture comprising the enzyme buffer, 2 μ*M* primer and 1.7 μ*M* [γ–^{3}P]ATP (Isotopchim, Ganagobie, France) and left at 37°C for 30 min.

PCR was carried out in a Crocodile III thermocycler (Appligène, Strasbourg, France) in 10 μl reaction mixture containing about 0.5 ng/μl template DNA, 20 p*M* radioactively labeled forward primer, 200 p*M* reverse primer, 1.5 m*M* $MgCl_2$, 0.7 m*M* dNTPs and 3 *U*/ml Taq polymerase (Promega, Madison WI, USA) in its buffer. Amplifications were achieved by running 30 cycles of 1.5 min at 94°C, 1 min at 46°C and 0.5 min at 72°C following a 3 min start at 94°C and terminated by 5 min at 72°C. The PCR products were heated at 95°C for 5 min in 33% formamide solution and electrophoresed in vertical gels of 0.4-mm thick 4% denaturing polyacrylamide / 0.5X TBE buffer for 4-5 hrs at 50 W. After migration, the gels were vacuum-dried at 80°C for 1 hr and autoradiographed against X-Omat film (Eastman Kodak, Rochester NY, U.S.A.). Allele sizes were obtained from sequences (Ohresser *et al.*, 1997; C. Daguin, P. Borsa and M. Ohresser, unpublished) or by log-linear interpolation on the gel autoradiographs. Size-homologies of alleles from different individuals were ascertained by side-by-side electrophoretic runs.

Correlations of alleles within individuals relative to the population, and within populations relative to the total population, were estimated using Weir & Cockerham's (1984) estimators f and θ, respectively. Random permutations on the matrices of individuals x genotypes were used to give the expected distributions of f and θ under the null hypotheses $f = 0$ and $\theta = 0$, respectively. The probability of occurrence of a parameter value larger or equal to the estimate was estimated as $P = (n+1)/(N+1)$ where n is the number of pseudo-values larger than or equal to the estimate and N is the number of random permutations (Sokal and Rohlf, 1995). The null hypothesis $f = 0$ was rejected when $P < 0.025$ (using a two-tailed test). The null hypothesis $\theta = 0$ was rejected when $P < 0.05$ (using a one-tailed test). For multiple tests we used the sequential Bonferroni adjustment as advocated by Rice (1989). The estimations of f and θ and permutation tests were made using Procedure FSTATS in the computer package GENETIX (Belkhir *et al.*, 1996).

3. Results

Long-range polyacrylamide gel electrophoresis revealed a high level of size polymorphism in *mac-1* Intron 1 (Fig. 2 and Table 1). Size alleles ranged from 250 bp to 315 bp as expected from preliminary data (Ohresser *et al.*, 1997; M. Ohresser, unpublished). Three size classes of alleles were designated by letters *a*, *b* and *c* as in Ohresser *et al.* (1997). Because of sharper resolution we detected size differences within each class leading to the recognition of 18 alleles in the present survey. Hence we chose to designate them by numbers following the letter formerly assigned to each class (*e.g. a1, b3, c4...*).

The distributions among samples of the allelic frequencies at locus *mac-1* are presented in Table 1. Alleles *a1, a4* and *a5* were characteristic of *M. edulis* since they were present in the *M. edulis* reference sample (GILL) and absent or virtually absent from the *M. galloprovincialis* reference sample (SETE). Similarly, alleles *b1, b2, c1, c2, c5, a2, a6,* and *a7* were considered as characteristic of *M. galloprovincialis*. *c2*, the most frequent allele in the reference *M. galloprovincialis* sample and which was absent in the reference *M. edulis* sample, was also the most common allele in the 3 samples from northwestern Africa. *a4* and *a5*, the most frequent alleles in Sample GILL, were virtually absent from SETE and from the northwestern African samples. The genotypes of the two mussels from Cansado (sample CANS) were *c1a8* and *c1c2*, therefore showing *M. galloprovincialis* characteristics. Incidentally, the Padstow mussel (sample POLZ) showed characteristics of both species with Alleles *c2* and *a5* as the most common. Although the frequencies of the latter were intermediate between those of the reference *M. edulis* and *M. galloprovincialis* populations, Allele *a4* was virtually absent in sample POLZ. *mac-1* allele frequencies in sample POLZ were generally intermediate between those of the reference *M. edulis* and *M. galloprovincialis* samples. This may reflect either extensive introgression in the POLZ population, or the admixture of differentiated populations or both. A closer look at genotype data is here necessary. By pooling, for convenience, all alleles characteristic of *M. edulis* (respectively *M. galloprovincialis*) into a synthetic allele "E" (respectively "G"), we obtained the following distribution of genotypes in sample POLZ: EE:10 ;

EG:20 ; GG:13 ; E-:6 ; G-:9 ; --:1 where dots refer to alleles that were absent in the reference samples (*b3, b4, c3, c4, a15* and *a8*). This genotype distribution suggests that the POLZ sample was drawn from an hybrid population (at least for *mac-1* genes) rather than consisting of a mixture of individuals with *M. edulis* and *M. galloprovincialis mac-1* genotypes.

Weir and Cockerham's (1984) *f*-estimates for each population ranged from *f* = 0.034 (TEMA) to *f* = 0.230 (GILL) (Table 2). None of these values was significantly different from 0 although they were all positive, thereby indicating some tendency towards heterozygote deficiency. Heterozygote deficiencies have been regularly reported in mussel populations (see Gaffney, 1994; Raymond *et al.*, 1997 for rewiews). Using the PCR amplification of nuclear DNA, one may overlook the occurrence of some alleles because of sequence polymorphism leading to mis-priming under given stringency conditions (see Hare *et al.*, 1996 for such an example of "null" alleles in oysters), hence artifactually increasing the frequency of apparent homozygotes in a sample. Two of the samples analysed in the present survey, POLZ and SETE (127 individuals) were also analysed by Ohresser *et al.* (1997) using a different pair of primers. Hence we were able to assess the probability of occurrence of null alleles due to priming site sequence polymorphism by comparing the genotypes obtained with our pair of primers (pair 1) with those obtained by Ohresser *et al.* (1997) (pair 2). All heterozygotes with Pair 1 (*N* = 58) remained heterozygotes for the same size-class alleles with Pair 2 and so did the homozygotes. No artifact caused by mis-priming was thus detected, leading to the conclusion that this type of null alleles is not the cause of heterozygote deficiency, if this actually occurs in the populations sampled in Polzeath and Sète.

Weir and Cockerham's (1984) θ-estimates, calculated for each pair of populations, are presented in Table 3. Each of the 3 northwestern African populations significantly differed from the reference *M. edulis* sample (GILL) (with θ ranging from 0.292 to 0.333) whereas no significant genetic heterogeneity was evident between each of the 3 northwestern African samples and the reference *M. galloprovincialis* sample (SETE) (with θ ranging from 0.009 to 0.026). However, when pooling all northwestern African individuals including those of Sample Cansado, a

significant albeit weak difference with the reference *M. galloprovincialis* was detected (θ = 0.013; *P* = 0.039). The Padstow mussel sample (POLZ) appeared differentiated from all the other populations (θ ranging from 0.054 to 0.152).

4. Discussion

Intron-1 length polymorphism at the actin gene locus *mac-1* proves useful as a genetic marker for population studies in *Mytilus* mussels. It is diagnostic between two mussel species, *M. edulis* L. and *M. galloprovincialis* Lmk and exhibits size-polymorphism within each species. The techniques reported in the present study allowed to discriminate between size-alleles differing from each another by as little as 1 bp leading to the recognition of 18 size-alleles in the total sample. Sequencing analysis (Ohresser *et al.*, 1997; C. Daguin, P. Borsa and M. Ohresser, unpublished) revealed that the large size differences observed between alleles are caused by the occurrence of several indels within the amplified fragments whereas small size differences generally correspond to single- or di-nucleotide gaps. With this and other nuclear DNA markers such as the calmoduline gene intron (Côrte-Real *et al.*, 1994a) and the gene encoding the byssal polyphenolic adhesive protein, *Glu* (Inoue *et al.*, 1995; Rawson *et al.*, 1996), an interesting array of population genetic markers is now available in *Mytilus*. PCR-based assays of genetic markers are also valuable because they are more sensitive than allozymes and can be used in the routine analysis of minute quantities of material, such as mussel larvae or spat (Côrte-Real *et al.*, 1994a,b; Rawson *et al.*, 1996; Bierne *et al.*, 1998).

The possible occurrence of "null" alleles that would be artifacts caused by mis-priming as reported for oysters (Hare *et al.*, 1996) was here tested on some samples by using two independent PCR-primer pairs. There was no evidence of such an artifact. The trend towards heterozygote deficiency that was apparent from genotype frequency distributions has therefore another cause (see Gaffney, 1994 and Raymond *et al.*, 1997, for in-depth discussions of this phenomenon). The high proportion in sample POLZ of heterozygotes with both an *M. edulis* and an *M. galloprovincialis*

mac-1 alleles indicates that the population it was drawn from is hybrid rather than a mixture of mussels from the two species. This result is compatible with the introgressed status of the mussel population at this location, that was reported by Beaumont *et al.* (1989). Nevertheless, the slight heterozygote deficiency in this sample, if it is real, may be interpreted as a Wahlund effect as already discussed in Ohresser *et al.* (1997).

We here showed that on the basis of allele frequencies at locus *mac-1* Intron 1, the mussel sampled on the northwestern African coasts are *M. galloprovincialis*, as formerly supposed on the basis of morphology and geographic location (Lubet *et al.*, 1984). To our knowledge, the occurrence of *M. galloprovincialis* on the Mauritanian coasts so far constitutes its southernmost reported location in the northeastern Atlantic.

No genetic heterogeneity was detected among the northwestern African samples including Tetouan. Although the latter location is at the eastern side of the Gibraltar strait, this sample did not exhibit genetic differences with the other northwestern African samples. There was an *a priori* reason for grouping the Tetouan sample with the other northwestern African samples as explained in the following. Tetouan is located in the Alboran Sea; because the Alboran Sea is considered to be a permanent intrusion of Atlantic surface waters in the Mediterranean, the Almeria-Oran oceanographic front which delimits it at the East (Tintore *et al.*, 1996) is thought to constitute the effective boundary between the Atlantic and the western Mediterranean waters. This physical structure is thought to constitute a barrier to larval flow, explaining the abrupt genetic change in allozyme and mitochondrial DNA haplotype frequencies between northeastern Atlantic-Alboran Sea and western Mediterranean *M. galloprovincialis* (Quesada *et al.*, 1995a,b; Sanjuan *et al.*, 1996). The θ-value at locus *mac-1*, between the northwestern African populations grouped together and the reference sample for *M. galloprovincialis* (θ = 0.013) was of the same order as the mean *Gst*-value reported for allozymes (*Gst* = 0.029 ± 0.032; Quesada *et al.*, 1995b) between each side of the Almería-Oran front. The genetic differentiation level observed using the two types of nuclear markers is therefore comparable and it contrasts with that reported for mitochondrial DNA (*Gst* = 0.128, calculated from

the haplotype frequency data in Sanjuan *et al.*, 1996). Such an observation is compatible with the hypothesis that the northeastern Atlantic and the western *Mediterranean M. galloprovincialis* populations have undergone geographic isolation. Under geographic isolation, mitochondrial DNA clones are indeed expected to evolve faster than nuclear DNA genes because of their smaller effective population size.

The present results, though preliminary, warrant further investigations on the evolutionary genetics of *Mytilus* populations using *mac-1* as a marker. Both *mac-1* polymorphism studies on Iberian populations and allozyme and mitochondrial DNA studies on northwestern African samples would be helpful for a more complete picture of the phylogeography of *M. galloprovincialis* in the northeastern Atlantic and the Mediterranean.

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Table 1

Allelic frequencies at locus *mac-1* Intron 1 in *Mytilus* spp. samples from Europe and northwestern Africa. Alleles were denominated according to the size class they belong to (*a*, *b* or *c*; see Ohresser *et al.*, 1997) and generally numbered from large to short with their size in base pairs (bp), deduced in part from sequencing (Ohresser *et al.*, 1997; C. Daguin, M. Ohresser and P. Borsa, unpublished) and also from their relative electrophoretic mobilities in denaturing 4% polyacrylamide gels. Abbreviations for samples as in Legend to Fig. 1. -, allele absent in the sample.

Allele (bp)	Sample					
	GILL	POLZ	SETE	TETO	TEMA	DAKH
b2 (317)	-	0.02	0.05	0.08	0.04	-
b1 (315)	-	0.06	0.21	-	0.08	0.09
b3 (313)	-	0.02	-	-	0.01	-
b4 (308)	-	-	-	-	-	0.03
c1 (294)	-	0.04	0.07	0.04	0.14	0.25
c2 (289)	-	0.29	0.54	0.58	0.50	0.47
c3 (288)	-	0.02	-	0.04	-	0.06
c4 (287)	-	0.02	-	-	0.01	-
c5 (277)	-	0.01	0.01	-	-	-
a1 (261)	0.02	0.03	-	-	-	-
a15 (257)	-	0.01	-	-	-	-
a2 (256)	-	0.08	0.01	0.12	0.01	-
a3 (255)	0.15	0.17	0.01	-	0.04	0.03
a4 (254)	0.40	0.02	-	0.04	-	0.03
a5 (253)	0.42	0.18	0.01	-	0.01	0.03
a6 (252)	-	0.02	0.08	0.08	0.10	-
a7 (251)	-	0.03	0.01	0.04	-	-
a8 (250)	-	0.01	-	-	0.05	-
(*N*)	(26)	(59)	(68)	(39)	(13)	(16)

Table 2

Weir and Cockerham's (1984) *f*-estimates in *Mytilus* spp. populations from the northeastern Atlantic and the western Mediterranean. Abbreviations for samples as in legend to Fig. 1

Sample	GILL	POLZ	SETE	TETO	TEMA	DAKH
f	0.230^{ns}	0.081^{ns}	0.086^{ns}	0.072^{ns}	0.034^{ns}	0.229^{ns}

^{ns}, not significant (1000 pseudo-samples obtained by permutations of alleles within each sample)

Table 3

Weir and Cockerham's (1984) θ-estimates between pairs of *Mytilus* spp. populations from the northeastern Atlantic and the western Mediterranean.

P	OLZ	SET	E	TETO	TE	MA	DAKH
GILL	0.152*	0.3	40*	0.333*	0.	307*	0.292*
POLZ		0.0	84*	0.067*	0.	058*	0.054*
SETE				0.021	0.	009	0.026
TETO					0.	005	0.000
TEMA							0.027

* values significantly different from 0 (1000 pseudo-samples obtained by permutations of genotypes in the total sample) with significance level adjusted using sequential Bonferroni procedure (Rice, 1989).

Figures legends

Fig. 1. Sampling localities for *Mytilus* sp. in northwestern Africa and for reference *M. edulis* and *M. galloprovincialis* in Europe. *GILL* Gilleleje, Denmark, Sep. 1996; *POLZ* Polzeath, south-west Britain, July 1996; *SETE* Sète, southern France, May 1996; *TEMA* Temara, Morocco, Nov. 1995; *TETO* Tetouan, Morocco, Nov. 1995; *DAKH* Dakhla, Western Sahara, Nov. 1995; *CANS* Cansado, Mauritania, Apr. 1996

Fig. 2. Length-polymorphism of the PCR products of Intron-1 of the actin gene *mac-1* in *Mytilus* spp. individuals. Allele size-classes are designated by letters *a*, *b*, or *c* followed by a number. The radioactively labeled PCR products of different individuals were loaded into different lanes of a vertical 4% denaturing polyacrylamide gel, electrophoresed and autoradiographed. Individual genotypes were the following : *lane A*, *c1c2; B*, *b2c2; C*, *a7a8; D*, *b1a4; E*, *c4c4; F*, *c3c4; G*, *c2a2; H*, *a1b1; I*, *a3a5; J*, negative PCR control; *K*, *a2a8; L*, *a2a7; M*, *c4a6; N*, *a2a5; O*, *a2a4*

Fig. 1 Daguin & Borsa

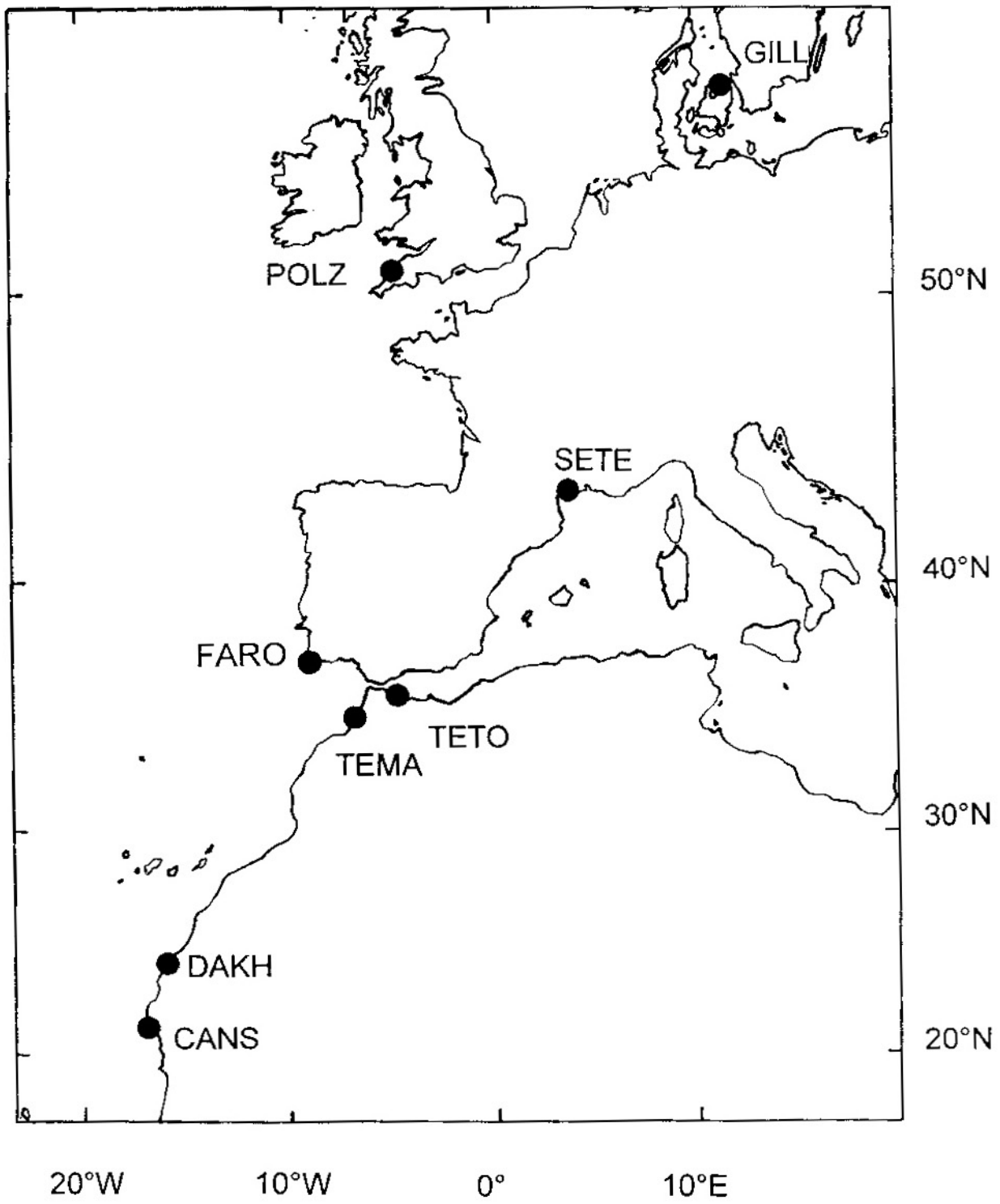
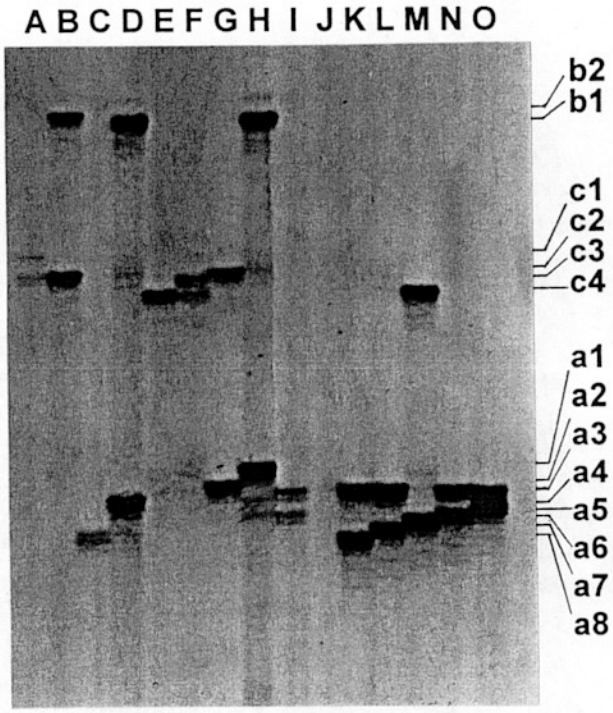
Fig. 2 Daguin & Borsa