Genetic divergence detected by ISSR markers and characterization of microsatellite regions in *Mytilus* mussels[i]

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Abstract

The wide distribution of microsatellites in mussels of the *Mytilus edulis* complex (Mytilidae) enables the analysis of inter-simple-sequence repeat (ISSR) markers. The aim of this investigation was to assess genetic differentiation in six sampling localities distributed along the European Atlantic coast to expose the potential of these markers in genetic studies requiring the detection of low polymorphism and as a source of sequences for developing microsatellite markers. We detected low genetic structuring within each member of the *Mytilus edulis* complex. Nei and Li distances and AMOVA clustered the individuals studied into two groups. On the basis of these results two sampling localities coming from the *M. edulis* × *M. galloprovincialis* mosaic hybrid zone in Western Europe were assigned to one species. On the other hand, mussels of a sampling locality in the Baltic Sea were not significantly different from a pure *M. edulis* locality supporting an extensive introgression of *M. edulis* in these individuals. Finally, 148 microsatellites were found in the sequences of 51 ISSR markers, and two polymorphic microsatellite markers were developed.

Keywords: Genetic variation; ISSR; microsatellite; mussel; *Mytilus*

Introduction

Choosing an effective method to assess genetic variability in a group of individuals is of great interest to many researchers studying population genetics. In recent years, different molecular markers based on PCR amplification have been developed and rapidly have become essential tools in this field. Some of these markers are microsatellite-based, such as the inter-simple sequence repeat (ISSR) markers (Zietkiewicz et al. 1994). ISSR markers are generated from nucleotide sequences located between two microsatellite priming sites inversely oriented on opposite DNA strands and near enough to be amplified by PCR (Fig. 1). This technique relies on the high polymorphism and wide distribution of microsatellites to detect low differentiation levels. Prior knowledge of the sequence is not needed, and numerous polymorphic bands are generated. Like other dominant markers, such as RAPDs, the ISSR amplification products are scored as present or absent without distinguishing between heterozygous and dominant homozygous. ISSR markers, however, have more stringent primer annealing conditions than RAPDs, which leads to higher reproducibility. Those features, along with the ease and cost, have brought attention to these markers.
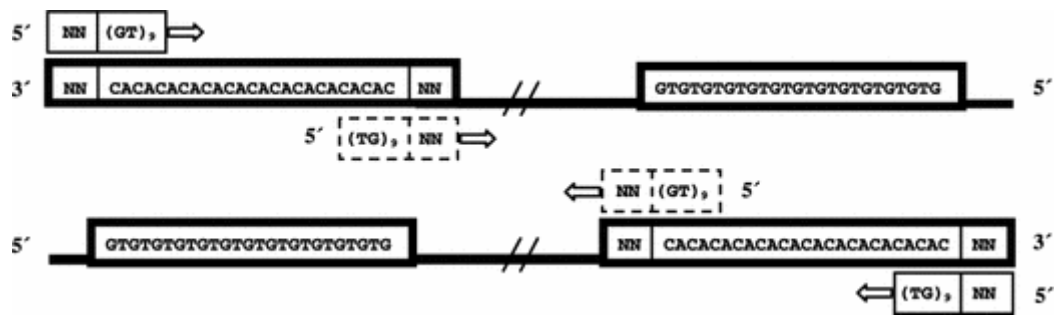
Figure 1. Structure of ISSR markers and annealing of anchored primers. Diagonal lines separate microsatellite priming sites inversely oriented on opposite DNA strands and near enough to be amplified by PCR. Dotted-line rectangles represent 3′ anchored primers, and small solid-line rectangles represent 5′ anchored primers. Arrows indicate direction of DNA polymerization (5′-3′). N represents an anchoring nucleotide

Research using ISSR markers has focused on evaluating genetic variation in terrestrial ecosystems. Less attention has been paid to the application of these markers in marine populations, where they have been used to evaluate the gene flow of two teleost species between the Red Sea and the Mediterranean (Hassan et al. 2003) and to detect a fine-scale genetic structure in the bivalve *Gemma gemma* (Casu et al. 2005). In the marine environment, natural borders between populations are less evident than in terrestrial ecosystems. In the case of *Mytilus* spp., the broad distribution, external fertilization, high fecundity, planktonic larval stage, and existence of hybrid zones between sibling species hinder the definition of population structure, the distribution limit of each species, and the parentage of a particular individual (Koehn 1991; Palumbi 1994). Different techniques have been applied to study genetic differentiation in the *Mytilus edulis* complex (a hybrid complex including *M. edulis*, *M. galloprovincialis*, and *M. trossulus*), but to date no ISSR studies have been published on this complex. The purposes of this study were, first, to study the pattern of genetic variation in the complex *Mytilus edulis* in several localities of the European Atlantic coast to expose the potential of these markers in genetic studies requiring the detection of low polymorphism; and second, to examine the suitability of ISSR markers as a source of sequences for developing microsatellite markers in these bivalves.

Materials and Methods

Sample Collection and DNA Extraction

The analysis of ISSR markers was performed on 120 *Mytilus* individuals from the European Atlantic coast. Mussels were obtained from the intertidal areas of Balcobo, Isle of Batz, Isle of Man, and Öland Island, and cultured mussels from Arousa and Yerseke. DNA was extracted as described by Winnepenninck et al. (1993) from 25 mg of adductor muscles and mantle. Samples stored in ethanol were rehydrated in PBS (0.137 M NaCl, 2.68 mM KCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4) and distilled water before DNA extraction. The geographic situation and number of individuals from each sampling locality are indicated in Fig. 2.

Figure 2. Geographic situation of each sampling locality. Sample sizes: Balcobo 22, Arousa 19, Isle of Batz 22, Yerseke 20, Isle of Man 18, Öland Island 19

Results obtained by Hummel et al. (2001) allowed us to identify the specimens from Yerseke as *M. edulis*, and individuals from Arousa and Balcobo were identified as *M. galloprovincialis* (Sanjuan et al. 1990). Baltic Sea mussels are considered hybrids *M. trossulus* × *M. edulis* as in Riginos and Cunningham (2005), who found that 96% of Baltic Sea mussels were hybrids. Finally, samples coming from the Isle of Batz and the Isle of Man could include *M. galloprovincialis*, *M. edulis*, or hybrids of both species, as both islands may be located in a mosaic hybrid zone (Bierne et al. 2003).

PCR amplification

Each PCR reaction of ISSR markers had a final volume of 25 μl, containing 20 ng of DNA template, 1× PCR buffer (16 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl, pH 8.8; 0.1% Tween-20), 1 μM primer, 0.2 mM each dNTP, 5.2 mM Mg_2Cl, and 0.75 U *Taq* DNA polymerase (Bioline). Amplifications were performed using a Touchdown PCR (Don et al. 1991) in a PTC-100 thermal cycler under the following conditions: 94°C 2 min, 94°C 20 s, 66°C 30 s, 72°C 2 min, and the annealing temperature was dropped 1°C for each of the subsequent 10 cycles followed by 30 cycles at 94°C 20 s, 55°C 30 s, and 72°C 2 min, with a final extension at 72°C 5 min. Primer ISSR-23 (Table 1) required a higher final annealing temperature, and as a result, only 6 step-down cycles were needed, and the annealing temperature was kept at 59°C for 36 cycles.

Table 1. ISSR primers used to analyze the *Mytilus* genome

Primer	Sequence[a]	Bands (bp)	Rp index[b]
ISSR-23	5′-YG(CA)$_9$-3′	725, 475, 250	1.59
ISSR-21	5′-YG(GA)$_9$-3′	1200, 1050, 950, 400	2.65
ISSR-18	5′-GATC(CAG)$_7$-3′	1425, 1275, 1050, 950, 725	3.13
ISSR-16	5′-YG(CT)$_9$-3′	1500, 1350, 1250, 900, 850	3.21
ISSR-13	5′-HVH(TTCG)$_4$-3′	1250, 950, 750, 625	2.84

[a]H: A, T, or C; V: C, G, or A; Y: C or T

[b]Rp: resolving power

Microsatellite loci subsequently discovered within ISSR markers (see below) were characterized in 22 mussels (*M. galloprovincialis*) from Balcobo, located on the Atlantic coast of Spain. Microsatellite fragments were amplified with the following conditions: 94°C 2 min, followed by 30 cycles of 92°C 1 min, T_a 1 min, and 72°C 30 s with a final extension step at 72°C 10 min. PCR reactions were carried out in a total

volume of 25 μl consisting of 20 ng DNA template, 1× Roche *Taq* PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl), 0.2 μM each forward and reverse primers, 0.2 mM each dNTP, 1.5 mM MgCl₂, and 0.75 U *Taq* DNA polymerase (Roche).

Electrophoresis and Band Scoring

Products obtained by DNA extraction and amplification of ISSR markers were observed on 1.5% agarose gels stained with ethidium bromide using the UVP Gelworks densitometry software, which quantifies DNA and assigns a fragment size to each band by scoring against a molecular weight marker (Roche 100 bp ladder). Distinct and reproducible bands were scored present (1) or absent (0), and a binary matrix was constructed. A negative control was added in each run to test for contamination. To choose scorable bands and ensure reproducibility, a group consisting of four samples of each locality was amplified and analyzed twice. Microsatellite markers were evaluated for consistent amplification and polymorphism using an Agilent 2100 Electrophoresis Bioanalyzer (Agilent Technologies) (Panaro et al. 2000).

Cloning

Pools of ISSR markers obtained by PCR were ligated into plasmid PCR2.1 Topo TA vector, used to transform *Escherichia coli* TOP10F′ competent cells (Invitrogen). Recombinant clones were selected as white colonies on ampicillin plates containing X-gal and IPTG. Inserts were sequenced using a capillary array electrophoresis sequencer CEQ 8000 Genetic Analysis System (Beckman Coulter).

Data Analysis

The usefulness of ISSR markers, as fingerprinting of the banding pattern obtained with each primer, was evaluated using the Rp index of Prevost and Wilkinson (1999), who found a strong relationship between this index and the ability of a primer to differentiate between individuals. The Rp of a primer is Rp = $\sum$Ib, where Ib (band informativeness) is equal to $1 - [2 \times (0.5 - p)]$, p being the proportion of individuals containing this band. This index could be helpful as a primer choice-criterion to perform fingerprinting analyses in a particular species. The value of this index for each primer does depend on the number of individuals. For these values to be comparable among different studies, however, the groups considered must have similar levels of genetic diversity and the studies must apply the same analytical procedure.

Differentiation measures were first calculated without considering the absence of a given band in two individuals to be due to an identical ancestral mutation and without assuming Hardy–Weinberg equilibrium. Nei and Li (1979) distances for all pairwise individuals were calculated from the binary matrix using the software RAPDplot 3.0 (Black 1998). The estimates of intergroup distances corrected for intraspecific variation were obtained as $D'_{ij} = D_{ij} - [(n_i - 1)D_i + (n_j - 1)D_j]/(n_i + n_j - 2)$, where D_i and D_j are the average distance in each group, D_{ij} is the average distance in all pairwise individual comparisons between groups i and j, and n_j and n_i are the number of individuals in each group (Kostia et al. 2000). The intergroup distance estimates were used to construct a neighbor-joining (NJ) tree. A principal coordinates analysis (PCA) was performed in SPSS 10.0.6 (SPSS Inc. 1999) using the Nei and Li distances (1979) generated between all pairs of individuals to determine how individuals from all localities cluster together.

The genetic structure was investigated using an AMOVA. The AMOVA analysis was carried out from a squared Euclidean distance matrix based on the binary matrix using the software Arlequin 2.0 (Schneider et al. 2000) to reveal the haplotypic diversity within localities, among localities, and among groups of localities.

To identify microsatellites in the ISSR markers, their sequences were analyzed with the program Tandem Repeats Finder 2.02 (TRF) (Benson 1999). This program uses an algorithm that finds simple sequences without requiring previous knowledge of the motif or size of the repetition unit. The search criteria were set

with default parameters and 20 as the minimum alignment score. Oligo 6.3 (Molecular Biology Insights) was used for designing primers to amplify microsatellites with enough flanking sequence.

Results

Anchored primers generated polymorphic banding patterns in *Mytilus* (Fig. 3). Five out of 24 3′ and 5′ anchored primers were selected according to clarity and reproducibility criteria (Table 1). Little variation was observed between the replicate band profiles, except for a few large (>1,500 bp) bands that were not reproducible. We selected 21 unambiguously scorable and reproducible markers. According to the Rp index, the primer ISSR-16 is the most appropriate to differentiate individuals, and a 250 bp band obtained with the primer ISSR-23 is the most useful to differentiate sampling localities (Table 1). This band was fixed in the Isle of Batz and near fixation in Balcobo (0.86) and Arousa (0.89), but in Yerseke, Isle of Man, and Öland Island it appeared in low frequency (0.10, 0.17, and 0.11, respectively).
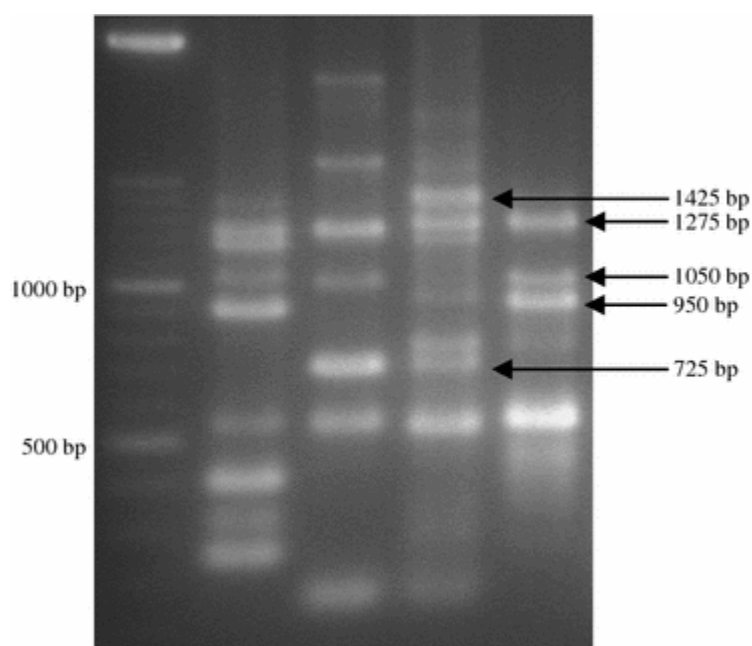
Figure 3. ISSR markers generated using the primer ISSR-18 with four *M. galloprovincialis* from Arousa. Sizes of selected bands are indicated on the right. The first lane contains a 100 bp ladder (DNA Molecular Weight Marker XIV, Roche)

An AMOVA was conducted on the binary matrix to appreciate the haplotypic diversity between groups of localities, between localities within groups, and within localities. Different preassigned structures were established, but it was the grouping (Balcobo, Arousa, Isle of Batz)/(Yerseke, Isle of Man, Öland Island) that displayed the higher proportion of variance between groups (13.24%, Φ_{CT} = 0.161). Very little of the variation was attributed to the locality (2.83%, Φ_{SC} = 0.033), and most of the variance appeared within localities (83.92%, Φ_{ST} = 0.132). The variance components were significant ($P < 0.001$) within each locality and between groups, showing the existence of heterogeneity at these levels. The variance calculated among localities of the same group suggests the existence of low genetic structuring (Φ_{SC} = 0.033, $P = 0.011$), using a null distribution computed by 1,000 permutations.

Locality clustering was performed from Nei and Li distances using the NJ method included in the software package Phylip 3.5c (Felsenstein 1993). Figure 4 shows the six sampling localities grouped into two clusters, one comprising Balcobo, Arousa, and the Isle of Batz and the other with Yerseke, Isle of Man, and Öland Island. These two major groups of individuals can also be observed in a PCA displayed on a two-dimensional plot (Fig. 5).

Figure 4. Unrooted NJ tree based on corrected Nei and Li distances as in Kostia et al. (2000)
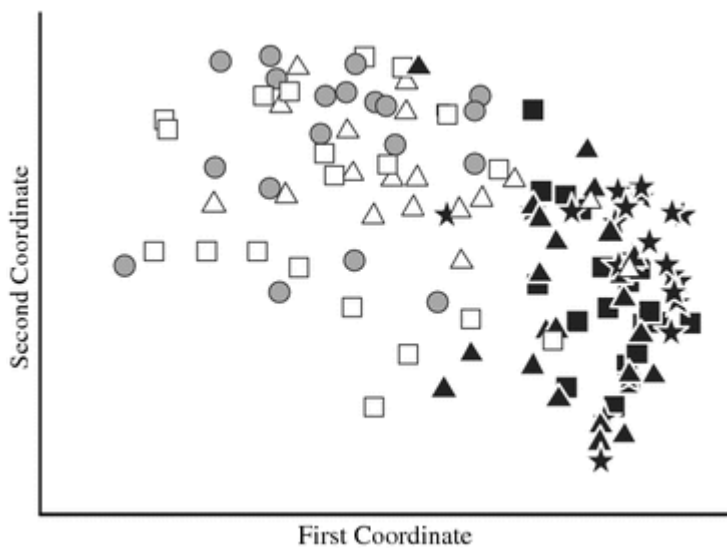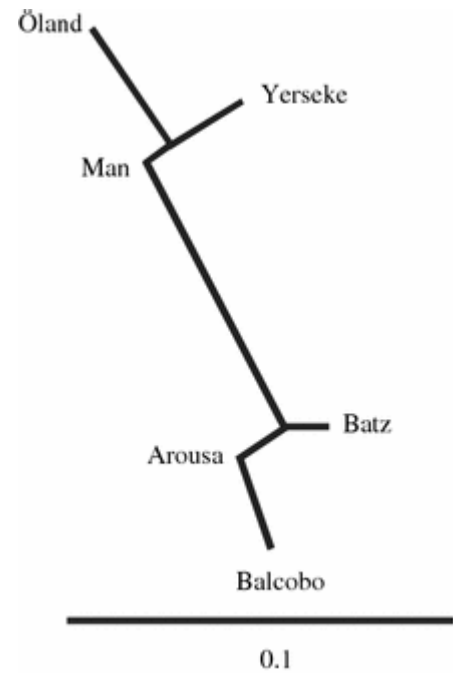
Figure 5. Principal coordinates analysis based on Nei and Li distances using 21 ISSR markers from 120 individuals. The percentage of total variance of each axis was 29.0% for the first axis and 12.6% for the second axis. ★, Balcobo ($n = 22$); ■, Arousa ($n = 19$); ▲, Isle of Batz ($n = 22$); □, Yerseke ($n = 20$); △, Isle of Man ($n = 18$); ◉, Öland Island ($n = 19$)

Fifty-one ISSR markers of *M. galloprovincialis* mussels from Balcobo were chosen randomly, cloned, and sequenced. The sequences were analyzed with the program Tandem Repeats Finder 2.02 (TRF) (Benson 1999), which found 148 microsatellites. Microsatellites were found at the 3′ and 5′ ends of each clone and 46 at internal positions (Table 2). Oligo 6.3 (Molecular Biology Insights) was used for designing primers to amplify the seven internal microsatellites with sufficient flanking sequence and more than five repeats. The Agilent 2100 Electrophoresis Bioanalyzer revealed the number of heterozygotes efficiently, allowing us to discard microsatellite loci with low polymorphism. Nevertheless, we detected an error in sizing fragments of approximately 5% between different runs of the same PCR reaction, similar to that described by Hierro et al. (2004); therefore, range sizes of alleles should be considered approximations. A sample of 22 *M. galloprovincialis* from Balcobo was analyzed. The results for the four primer pairs that yielded amplification products are shown in Table 3. One proved to be monomorphic (*Mg192*), and another (*Mg272*) generated too many nonspecific bands and was judged unacceptable for analysis. Two microsatellites (*Mg181* and *Mg220*) generated scorable and polymorphic products. Observed heterozygosities at *Mg181* and *Mg220* were 0.76 and 0.78, respectively.

Table 2. Description of the microsatellites found in 51 ISSR markers

ISSR primer[a]	Size	Repetitive motifs found along the sequence[b]	Accession no.
YG(CA)$_9$	681	(CA)$_9$ (ATTT)$_5$ (TA)$_7$ (TG)$_{12}$	AJ938137
	347	(AC)$_{17}$ (CAAC)$_3$ (CAAT)$_3$ (CA)$_{19}$ (TG)$_{10}$	AJ938136
	236	(CA)$_9$ (AT)$_5$ (TG)$_{51}$	AJ938135
YG(GA)$_9$	629	(GA)$_{10}$ (GTCC)$_7$ (TC)$_9$	AJ938134
	622	(GA)$_9$ (TC)$_5$ (TC)$_9$	AJ938133
	573	(GA)$_{13}$ (TC)$_9$	AJ938132
	432	(GA)$_9$ (TC)$_5$ (TC)$_9$ (TC)$_9$ (TC)$_9$	AJ938131
	393	(GA)$_9$ (TC)$_9$ (TC)$_5$ (CT)$_{27}$	AJ938130
GATC(TCTG)$_8$	775	(TCTG)$_8$ (TCCG)$_4$ (ACAG)$_8$	AJ938129
	680	(TCTG)$_8$ (CGGA)$_4$ (ACAG)$_7$	AJ938128
	570	(TCTG)$_8$ (CAGA)$_8$	AJ938127
	415	(TCTG)$_8$ (CAGA)$_{10}$	AJ938126
GATC(CGT)$_7$	744	(CGT)$_9$ (GAC)$_7$	AJ938125
	729	(CGT)$_8$ (GAC)$_7$	AJ938124
	729	(CGT)$_8$ (GAC)$_7$	AJ938124
	617	(CGT)$_7$ (ACG)$_7$	AJ938123
	483	(CGT)$_{11}$ (ACG)$_{10}$	AJ938122
	457	(CGT)$_{12}$ (ACG)$_7$	AJ938121
	407	(CGT)$_7$ (ACG)$_6$	AJ938120
	157	(CGT)$_9$ (ACG)$_4$ (GAT)$_4$ (ACG)$_7$	AJ938119
	149	(CGT)$_{15}$ (ACG)$_7$	AJ938118
YG(CT)$_9$	628	(CT)$_9$ (AG)$_{11}$	AJ938117
	564	(CT)$_9$ (GA)$_9$	AJ938116
	554	(CT)$_{10}$ (TTCT)$_4$ (AG)$_5$ (GA)$_5$ (AG)$_{18}$	AJ938115
	471	(CT)$_9$ (GA)$_9$	AJ938114
	334	(CT)$_9$ (GA)$_5$ (GA)$_9$	AJ938113
	252	(CT)$_{10}$ (AG)$_5$ (AG)$_9$	AJ938112
KRV(CT)$_6$	715	(CT)$_6$ (GA)$_6$	AJ938111
	650	(CT)$_8$ (GA)$_6$	AJ938110
	564	(CT)$_{11}$ (TTG)$_5$ (AG)$_6$	AJ938109
	498	(CT)$_7$ (CTTTTA)$_6$ (AGTGAG)$_3$ (GA)$_5$ (GA)$_{24}$ (GT)$_6$ (GA)$_6$	AJ938108
	433	(CT)$_6$ (GA)$_6$	AJ938107
	343	(CT)$_6$ (CT)$_5$ (AG)$_8$	AJ938106
	274	(CT)$_6$ (AC)$_5$ (GA)$_{11}$	AJ938105
	206	(CT)$_6$ (AGAA)$_3$ (ATCG)$_5$ (GA)$_5$ (AG)$_6$	AJ938104
	173	(CT)$_{14}$ (TCTT)$_3$ (AG)$_6$	AJ938103
	167	(CT)$_9$ (TCGA)$_3$ (TCTT)$_3$ (AG)$_6$	AJ938102
HVH(TTCG)$_4$	736	(TTCG)$_4$ (ACGA)$_{10}$	AJ938101
	655	(TTCG)$_4$ (CGAA)$_4$	AJ938100
	506	(TTCG)$_6$ (CGTC)$_8$ (ACGA)$_4$	AJ938099
	465	(TTCG)$_{15}$ (CGTC)$_5$ (ACGA)$_4$	AJ938098
	333	(TTCG)$_6$ (AT)$_5$ (AACG)$_4$	AJ938097

	288	(TTCG)$_7$ (ACGA)$_4$		AJ938096
	193	(TTCG)$_4$ (ACGA)$_4$		AJ938095
HVG(TG)$_7$	312	(GT)$_8$ (CA)$_8$		AJ938094
	190	(GT)$_8$ (CA)$_7$		AJ938093
KKVRVRV(CT)$_6$	578	(CT)$_6$ (CT)$_{10}$ (TCTCAC)$_{13}$ (TCACTC)$_3$ (TCACAT)$_7$ (AG)$_5$ (GA)$_8$		AJ938092
	404	(CT)$_6$ (CT)$_6$ (AG)$_8$		AJ938091
	328	(CT)$_6$ (AG)$_9$		AJ938090
	269	(CT)$_6$ (CT)$_6$ (AG)$_{11}$		AJ938089
	185	(CT)$_6$ (AG)$_7$		AJ938088
	148	(CT)$_{10}$ (GA)$_6$		AJ938087

[a] Y = C/T, K = G/T, R = A/G, V = A/C/G, H = A/C/T

[b] Core motifs of six or fewer nucleotides tandemly repeated at least three times

Table 3. Characterization of four microsatellite markers in the mussel *Mytilus galloprovincialis*

Locus	EMBLEM no.	Repeat motif	T_a	Primer sequence (5'-3')	H_o	Comments
Mg181	AJ938098	(CGTC)5	56	F: CTGCTTCAGGTTTTATGTCC	0.7727	Polymorphic
				R: TCTGACAAATTGGCTTTTAAT		
Mg192	AJ938136	(CA)$_{19i}$[a]	56	F: TGAGCACATCATCCTGAATA	0	Monomorphic
				R: CCACACACATGCAATGATAT		
Mg220	AJ938108	(GA)$_{24}$	56	F: CCATGCTAGTATGAGGGTGCTAGTG	0.4545	Polymorphic
				R: CTAACACTCTCTCACACAACGGCAT		
Mg272	AJ938130	(TC)$_9$…(TC)$_5$	58	F: CCTCTAACACTAGGCCTCACCT	–	Unscorable
				R: AGTGCCAGTGTGAGAGTGTGAG		

Note: *T a*, annealing temperature; *H o*, observed heterozygosity

[a] Imperfect tandem repeats

Discussion

Assessment of Genetic Differentiation

Due to the widespread distribution of microsatellites in eukaryotic genomes, the amplification of ISSR markers does not require previous knowledge of the sequence that is to be analyzed. Nevertheless, the potential of the technique depends on the variety and frequency of microsatellites, which show considerable variance among species (Dieringer and Schlötterer 2003). This study provides evidence for the usefulness of ISSR markers to analyze genetic variability in *Mytilus*.

Inter-simple sequence repeat markers showed high genetic variation in *Mytilus*. In large populations, the probability of fixation or loss of alleles caused by genetic drift is more reduced than in small populations. Therefore, assuming mutation-drift equilibrium in ISSR markers and the intrinsic instability of microsatellite-rich sequences, the large populations of mussels accumulate neutral variants, maintaining high genetic diversity.

The variability revealed by ISSR markers identified each individual as a different haplotype. The detection of numerous polymorphic bands that provide information about several loci simultaneously indicates that this technique could be useful to define parentage and relatedness in mussels. Individual identification can provide a direct estimate of differential reproductive success of a group of individuals in a hatchery.

Variance in reproductive success has been detected in molluscs (Hedgecock 1994) and produces a progeny that does not maintain the genetic composition of the broodstock. This reduction in genetic variability would make a selective breeding program difficult, and both ISSR markers and the Rp index as a primer choice criterion to assess identification and parentage relations could help to monitor this phenomenon.

The analysis of genetic differentiation among sampling localities using Nei and Li distances and the AMOVA grouped individuals into two clusters. One included Balcobo, Arousa, and Isle of Batz and the other Yerseke, Isle of Man, and Öland Island. This separation of individuals into two groups, however, was not well-defined (Fig. 5). This distribution could be explained by gene flow, cohabitation, or hybridization of two species in the same place.

The existence of the hybrids *M. edulis* × *M. galloprovincialis* and *M. edulis* × *M. trossulus* has been extensively reported and led some authors to consider these taxa as ecotypes of *M. edulis* (Gosling 1992). For example, the Atlantic coast of France and the British Isles are hybrid zones with contiguous regions rich in either *M. galloprovincialis* or *M. edulis* and where hybrids are also present. The grouping of the Isle of Batz with Arousa and Balcobo, identified as pure *M. galloprovincialis* by allozymes and morphometry (Sanjuan et al. 1990), indicates that the samples from the Isle of Batz could be located in a *M. galloprovincialis*-rich region. Notably, the marker that differentiates Arousa and Balcobo better from the pure M. edulis locality of Yerseke (Hummel et al. 2001), a 250 bp band amplified by primer ISSR-23, is fixed in the Isle of Batz. On the other hand, our data suggest that the Isle of Man is in a *M. edulis*-rich zone, as this sample groups with Yerseke. Besides, the PCA did not separate individuals from the Baltic Sea and the mussels originated from Yerseke and Isle of Man. According to allozyme data, the distribution of *M. edulis* in Europe spreads from the Vizcaya Gulf to the north of the Polar Arctic Circle, but *M. trossulus* is confined in the Baltic Sea (Varvio et al. 1988). Analyses of nuclear (Borsa et al. 1999; Riginos et al. 2002) and mitochondrial markers (Wenne and Skibinski 1995) in mussels from the Baltic Sea, however, have revealed a high introgression of *M. edulis* alleles in Baltic Sea mussels. This high introgression seems to be strong enough in Öland Island to cluster this locality together with M. edulis, although the species *M. edulis* and *M. galloprovincialis* are more closely related (Rawson and Hilbish 1995, 1998; Hoeh et al. 1997; Hilbish et al. 2000).

Characterization of Microsatellites

Sequence analysis of 51 ISSR markers revealed that each sequence contained a microsatellite at both ends of the insert and many of them also had internal ones. The presence of these additional sequences in *Mytilus* supports the hypothesis previously reported in other organisms, in agreement with the clustering of microsatellites in some genomic regions (Schlötterer 2000).

High inherent instability and codominance make microsatellites extremely useful as genetic markers. In view of the microsatellites found in ISSR markers, sequencing of ISSR markers provides data that can be used to amplify single microsatellite loci. In previous studies on molluscs, microsatellites have been isolated from genomic libraries, and then these sequences have been useful in natural populations (Astanei et al. 2005) or as a tool of parentage identification (Walker et al. 2005). The development of microsatellite markers is a long and expensive process, however, and so some researchers examine the applicability of markers from related species (Evans et al. 2001). The success of these studies is clearly related to phylogenetic distance between species. Furthermore, few loci are available in mussel-related species, hence the isolation and characterization of microsatellite markers from the sequences amplified by anchored primers represents a feasible alternative as described previously (Fisher et al. 1996; Keiper et al. 2006; Varela et al. 2007).

In summary, samples coming from the Isle of Man and the Isle of Batz, located in the *M. edulis* × *M. galloprovincialis* hybrid zone in Western Europe, proved to be rich in *M. edulis* and *M. galloprovincialis*, respectively. The mussels of Öland Island were not significantly different from a pure *M. edulis* locality (Yerseke), suggesting a high introgression of alleles of *M. edulis* in these individuals. ISSR markers detected high variability within each sampling locality and low genetic structuring within each member of the *Mytilus edulis* complex from the Atlantic European coast. This technique provided a fast and cost-effective analysis of polymorphism in a group of individuals, and at the same time, sequence data were gathered to perform more studies using single microsatellite loci.

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