# Temporal genetic variation of microsatellite markers in the razor clam *Ensis arcuatus* (Bivalvia: Pharidae)[i]

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## Abstract

The aim of this study was to characterize new microsatellite loci in the razor clam *Ensis arcuatus* (Bivalvia: Pharidae) and examine the temporal genetic variability of a natural bed in Cies Islands (Galicia, north-western Spain) exploited by apnoea divers and affected by the 'Prestige' oil tanker spill in November 2002. In this work, we characterized four polymorphic microsatellites using an alternative approach that relies on the amplification and sequencing of ISSR markers. Observed heterozygosities ranged from 0.1053–0.6800 and number of alleles from 4–19. Linkage equilibrium was observed in all loci and two of them showed significant deviations from Hardy–Weinberg equilibrium. Estimators of FST between samples were low (<0.05) and not different from zero with a confidence level of 5%. We did not detect a clear decreasing tendency in genetic diversity although we found a significant change in allelic frequencies among samples (P = 0.0024) after the 'Prestige' oil spill. We propose that both phenomena could be related to a high variance in genetic success and/or a movement of adults or larvae from different source populations.

**Keywords:** *Ensis arcuatus*, genetic diversity, microsatellites, 'Prestige' oil spill, razor clam

## Introduction

The razor clam *Ensis arcuatus* (Jeffreys, 1865) (Bivalvia: Pharidae) forms natural beds in intertidal and shallow sublittoral waters along the European Atlantic coast from Norway to Spain including the coast of the British Isles (Hayward & Ryland, 1998). *Ensis arcuatus* is the razor clam with the highest commercial value in European markets (according to the Eurostat information database). In the national park of Islas Atlánticas (Cies Islands, north-western Spain) this resource is only extracted by apnoea divers in contrast to other regions of Europe where hydraulic dredging is allowed (Robinson & Richardson, 1998; Gaspar *et al.*, 1999; Hauton *et al.*, 2007).


**Figure 1**. Location of the natural bed of razor clams *Ensis arcuatus* in Cies Islands (Galicia, north-western Spain).

In November 2002, the 'Prestige' oil tanker was wrecked 120 miles off north-western Spain spilling about 63,000 tons of heavy fuel oil (Figueras *et al.*, 2005). The spill affected a great extension of shores and a thick deposit of fuel arrived at the marine bottoms that *Ensis arcuatus* inhabits, affecting the Cies Islands extensively. The effects of an oil spill can differ among species. For instance, the 'Erika' oil spill produced a high mortality in sea urchins (Barillé-Boyer *et al.*, 2004), while no measurable effect was found in dolphins and seals (Ridoux *et al.*, 2004). Previous studies on the effects of oil spills on *Ensis* showed high levels of mortality after the wreckage of 'Torrey Canyon' (Smith, 1968) and 'Braer' (Glegg & Rowland, 1996) in Great Britain, and the 'Amoco Cadiz' in Brittany (Southward, 1978), and it is considered to be a species very intolerant to the presence of hydrocarbons in the water. Therefore, it is possible that the 'Prestige' oil spill could have affected populations of razor clams.

After a period of time populations affected either by overexploitation or a major pollution event may eventually recover their size. However, even apparently fully recovered populations in terms of size or DNA damage could have a low capability of adaptation to environmental changes if they suffered a genetic diversity loss by a population bottleneck (Lande & Shannon, 1996). This low capability of adaptation could have disastrous consequences for the survival of the population. Furthermore, the analysis of genetic diversity plays an important role in the management of a stock (Ward, 2006). The most straightforward approach to measure the genetic diversity of a population is to produce estimates of the allelic richness and

heterozygosity of this group of individuals expecting a significant decrease of these estimates in case of an important reduction in effective population size (Nei, 2005).

Recently, progress in our understanding of the biology of this species has focused on development and reproduction (Da Costa *et al.*, 2008), pathology (Darriba *et al.*, 2006) and cytogenetics (Fernández-Tajes *et al.*, 2008). Nevertheless, despite the importance of *Ensis arcuatus* in the intertidal and shallow sublittoral ecosystem, and the interest from the fishing industry, little is known about the population structure and genetic variability of this species. Because microsatellite markers are highly polymorphic, they can be useful to properly manage natural stocks and design effective breeding programmes managing differences in reproductive success and taking advantage of hybrid superiority, phenomena which are widely reported in marine bivalves (Gaffney, 1994; Hedgecock, 1994). The results of the present investigation constitute the first report on the genetic background of natural populations of the razor clam *Ensis arcuatus*. The aim of this study was to develop microsatellite markers and study the temporal genetic variability of a natural bed exploited by apnoea divers and affected by the 'Prestige' oil tanker spill to test for a reduction in genetic variability.

## Materials and methods

Specimens were obtained from a natural bank off the Cies Islands located in north-western Spain (42°13[variant prime]N 8°54[variant prime]W) (Figure 1) on four dates (25 January 1999, N = 25; 18 September 2003, N = 23; 19 January 2004, N = 26; 10 November 2004, N = 34). 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. DNA was extracted as described by Winnepenninckx *et al.* (1993) from 25 mg of adductor muscles and mantle tissues.

Microsatellites were isolated from the sequences of inter-simple sequence repeat (ISSR) markers (Fisher *et al.*, 1996; Francisco-Candeira *et al.*, 2007) as described in Varela *et al.* (2007). We amplified ISSR markers using the following anchored primers: $YG(CA)_9$, $YG(GA)_9$, $GATC(CAG)_7$, $GATC(CGT)_7$, $YG(CT)_9$, $G(CT)_9$, $KRV(CT)_6$, $HVH(TTCG)_4$, $HVG(TG)_7$, and $KKVRVRV(CT)_6$. PCR amplification of ISSR markers was conducted using a touchdown protocol: initial denaturation 94°C (2 minutes), followed by 94°C (20 seconds), 66°C (30 seconds), and 72°C (2 minutes). The annealing temperature was dropped 1°C for each of the subsequent ten cycles, followed by 30 cycles at 94°C (20 seconds), 55°C (30 seconds), and 72°C (2 minutes), with a final extension at 72°C (5 minutes). Amplification of the ISSRs was carried out in 25 ml of 1 x $NH_4$ reaction buffer (16 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl, pH 8.8; 0.1% Tween-20), 1 µM primer, 0.2 mM of each dNTP, 5.2 mM $Mg_2Cl$, 0.75 U *Taq* DNA polymerase (Bioline), and 20 ng of genomic DNA. Pools of ISSR markers amplified using each of the anchored primers were cloned following the PCR2.1 TOPO® TA (Invitrogen) protocol. DNA from 52 positive clones was sequenced using a capillary array electrophoresis sequencer CEQ 8000 Genetic Analysis System (Beckman Coulter). Sequences were screened with the program Tandem Repeats Finder 2.02 (TRF) (Benson, 1999) to identify microsatellites. Detailed analysis of 52 ISSR markers of *Ensis arcuatus* revealed the presence of 183 microsatellites (EMBL Accession numbers AM491138-AM491187). Each sequence contained a microsatellite at 3[variant prime] and 5[variant prime] ends of the insert, and 22 also had internal ones. These results allowed the design of ten primer pairs to amplify internal microsatellites with enough flanking sequence using the software Oligo 6.3 (Molecular Biology Insights).

Microsatellite fragments were amplified under the following conditions: 94°C (2 minutes), followed by 30 cycles at 92°C (1 minute), $t_a$ (1 minute), and 72°C (30 seconds) with a final extension step at 72°C (10 minutes). PCRs were carried out in a total volume of 25 [mu]l containing 0.2 µM each of forward and reverse primers, 0.2 mM dNTPs, 2-2.4 mM $MgCl_2$, 0.75 U Roche *Taq* DNA polymerase, and 20 ng DNA

template. An Agilent 2100 Electrophoresis Bioanalyzer (Agilent Technologies) was used to carry out an initial screening of the ten primer pairs, four of which were scorable and polymorphic (Table 1). Reverse primers were 5[variant prime]-labelled with fluorescent dyes (WellRED oligos, Proligo) and then amplification products were electrophoresed in a CEQ 8000 Genetic Analysis System apparatus (Beckman Coulter).

*Genepop* version 3.3 (Raymond & Rousset, 1995) was used to calculate observed and expected heterozygosities and test for Hardy-Weinberg equilibrium (HWE) and genotypic linkage disequilibrium. Fisher exact tests were also carried out with this software to test if there was temporal differentiation of the allele frequencies. We used Micro-Checker (Oosterhout *et al.*, 2004) to obtain estimates of the null allele frequencies according to the first estimator of Brookfield (1996). Afterwards, allelic variation of these markers was examined and compared among sampling localities using FSTAT Version 2.9.3 (Goudet, 2001). Estimates of the allelic richness (A) were corrected for differences in sample size by rarefaction (Leberg, 2002). Subsequently, changes in genetic diversity were tested by a Friedman test using the program SPSS version 15 (SPSS Inc, Chicago, Ill). Estimators of $F_{ST}$ (Weir & Cockerham, 1984) between samples were calculated using Genetix (Belkhir *et al.*, 2003).

**Table 1.** Characterization of four microsatellite markers in 108 *Ensis arcuatus* razor clams.

| Locus | EMBL No. | Motif (5'-3') | Primer sequence (5'-3') | [Mg $Cl_2$] | $T_a$ | Alleles | Sizes (bp) |
|---|---|---|---|---|---|---|---|
| Ea138 | AM491165 | $(TCTG)_6$ | F: CGGTCTAGTTCGTTGTGCTTCG | 2.4 | 55 | 8 | 123-163 |
| | | | R: ACCTCGGTGACCTTCACCTGAG | | | | |
| Ea163 | AM491178 | $(GAT)_{14}$ | F: CAGCAACAACATCATTTTCGA | 2 | 52 | 19 | 126-177 |
| | | | R: ATCGCCATCAGATCACCATTA | | | | |
| Ea219 | AM491161 | $(GA)_{26}$ | F: GGGATCGAGCGCTAGTAGGTA | 2.4 | 58 | 13 | 207-233 |
| | | | R: CGTATAGTTTATGGCTGTTTCTATCAA | | | | |
| Ea269 | AM491163 | $(CA)_6$ | F: CGGTATATGCGTGGTTAGACCAC | 2.4 | 55 | 4 | 269-275 |
| | | | R: GAACCACGCTAGTTTCTTGGTGA | | | | |

$T_a$, annealing temperature.

## Results and discusion

We detected 4 to 19 alleles per locus (Table 1) and observed heterozygosities ($H_O$) ranging from 0.1053 to 0.6800 (Table 2). All markers were unlinked and two (Ea138 and Ea163) conformed to HWE at a 5% significance level in all samples. Even after applying Bonferroni correction for two loci (Ea219 and Ea269), significant deviations from HWE were observed due to the deficiency of heterozygous genotypes ( $P < 0.01$) in at least one sample (Table 2). These results suggest that the markers Ea219 and Ea269 may have null alleles because of the deficit of heterozygous genotypes. With the marker Ea219 the estimates of the null allele frequencies varied from 0.0907 on 10 November 2004 to 0.1304 on 18 September 2003. And in the marker Ea269 the estimates varied from 0.1180 on 10 November 2004 to 0.1975 on 19 January 2004.

**Table 2.** Heterozygosity ($H_O$ observed, $H_E$ expected) in each locus and sample

| | **Ea138** | **Ea163** | **Ea219** | **Ea269** |
|---|---|---|---|---|
| **01/25/99** | | | | |
| $H_O$ | 0.5417 | 0.6522 | 0.4167 | 0.1053* |
| $H_E$ | 0.6606 | 0.5151 | 0.5764 | 0.5485 |
| **09/18/03** | | | | |
| $H_O$ | 0.5217 | 0.6522 | 0.3043* | 0.2857 |
| $H_E$ | 0.6957 | 0.518 | 0.5 | 0.4717 |
| **01/19/04** | | | | |
| $H_O$ | 0.5313 | 0.5455 | 0.1379* | 0.3214 |
| $H_E$ | 0.688 | 0.4559 | 0.2515 | 0.4994 |
| **11/10/04** | | | | |
| $H_O$ | 0.68 | 0.6154 | 0.2692* | 0.2174* |
| $H_E$ | 0.756 | 0.5096 | 0.4209 | 0.5019 |

*, significant departures from Hardy-Weinberg equilibrium after Bonferroni correction ($P < 0.0125$).

The results of the genetic diversity analysis are presented in Table 3. This table shows some changes in genetic diversity both in terms of allelic richness and heterozygosity for different samples. Nevertheless, we did not find a clear decreasing tendency in genetic variability due to the 'Prestige' oil spill or the exploitation of this species. Moreover, these changes in genetic diversity were not significant by a Friedman test. Concordantly, Piñeira *et al.* (2008) did not find a significant overall reduction in genetic diversity in the marine snail *Littorina saxatilis* after the 'Prestige' oil spill. Our results suggest that the population either suffered a very low intense bottleneck and/or had recovered with individuals coming from closed unaffected areas. Interestingly, recent studies on the effects of the 'Prestige' oil spill on mussels *Mytilus galloprovincialis* found that oil-exposed mussels had a higher DNA damage (Laffon *et al.*, 2006) and a lower growth (Peteiro *et al.*, 2006) than reference mussels although no effects were found two years after the wreckage (Cajaraville *et al.*, 2006; Peteiro *et al.*, 2008).

**Table 3.** Allelic richness in each locus (A, average allelic richness per sample) and heterozygosity ($H_O$ observed, $H_E$ expected)

| | **25/01/99** | **18/09/03** | **19/01/04** | **10/11/04** |
|---|---|---|---|---|
| **Ea138** | 4.667 | 5.609 | 5.799 | 4.903 |
| **Ea163** | 7.308 | 8.203 | 10.084 | 6.254 |
| **Ea219** | 7.326 | 8.332 | 6.075 | 4.478 |
| **Ea269** | 4 | 2.999 | 2.87 | 2 |
| **A** | 5.825 | 6.286 | 6.207 | 4.409 |
| $\boldsymbol{H_O}$ | 0.429 | 0.441 | 0.402 | 0.384 |
| $\boldsymbol{H_E}$ | 0.575 | 0.546 | 0.547 | 0.474 |

Estimators of $F_{ST}$ (Weir & Cockerham, 1984) between samples were low (<0.05) and did not differ from zero with a level of significance of 5% and Fisher exact tests did not detect any significant genotypic differentiation for each population pair. However, Fisher exact tests detected a significant change in allelic frequencies among samples ( $P = 0.0024$), this change brought about by loci Ea279 ($P = 0.0217$) and Ea163 ($P = 0.0082$). Genetic differences among samplings of the same locality could be caused by changes in the genetic composition from one cohort to another because of a high variance in genetic success (Hedgecock, 1994) or by a movement of adults or recruitment from different source populations. Stochastic differentiation has been described in marine invertebrates such as clams (David *et al.*, 1997; Vadopalas *et al.*, 2004), oysters (Li & Hedgecock, 1998), and sea urchins (Moberg & Burton, 2000). In relation to this, Fahy & Gaffney (2001) indicated that beds of razor clams can move massively to find better conditions and an appropriate substrate.

Contamination produced by the 'Prestige' oil spill was very extensive but heterogeneous, alternating intensely affected zones with non-affected. Our results are consistent with this heterogeneity in the distribution of oil spill and with the reproductive and ethological features of *Ensis arcuatus*. This species can produce a great number of offspring with a prolonged larval pelagic life span, which would allow non-affected larvae and adults to occupy a substrate in a short period of time. Presumably, these features contributed to prevent razor clam exploitation to be very affected by the oil spill. Figure 2 shows the catches of razor clams in Galicia (north-western Spain) according to the data obtained from the *Plataforma Tecnolóxica da Pesca* information database (www.pescadegalicia.com). In this figure it can be seen that the catches decreased after the 'Prestige' oil spill for two years, in part due to the closure of beds to exploitation in the months following the oil spill. Nevertheless, in the last years annual production has almost doubled since before the spill.

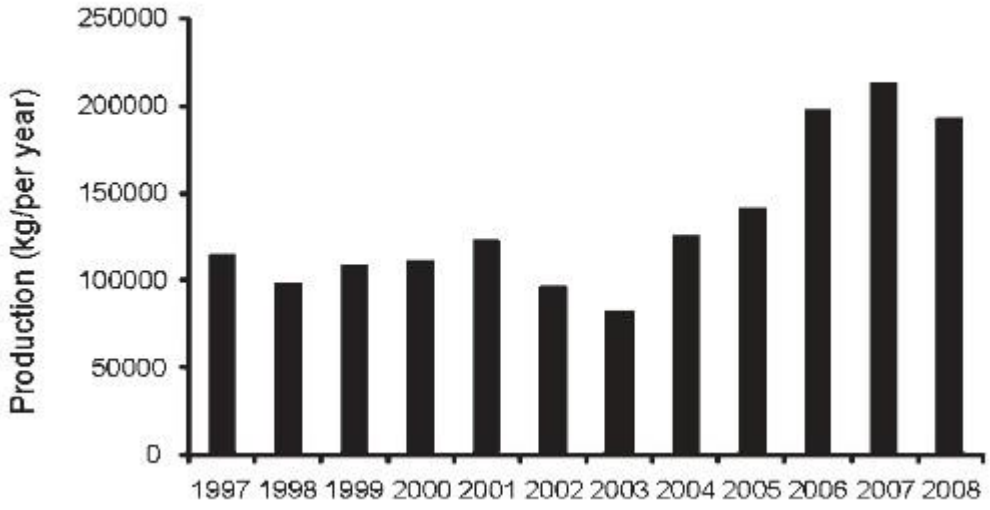


**Figure 2.** Production of razor clams *Ensis arcuatus* (kg per year) in Galicia (north-western Spain) before and after the 'Prestige' oil spill in November 2002.

To sum up, the microsatellite markers developed here will be valuable for future studies on the population structure of *Ensis arcuatus* in the wild and the aquaculture of this species to monitor the exploitation of razor clams, and study if the actual rate of exploitation is sustainable. Furthermore, we found a significant change in allelic frequencies among samples after the 'Prestige' oil spill. We propose that this change in allelic frequencies and the fact that we could not detect a decrease in genetic diversity could be related to a high variance in genetic success and/or a movement of adults or larvae from different source populations. The analysis of these markers suggested the existence of a temporal allelic change in our samples, and encourages future examinations involving sequencing and parentage analysis to avoid attributing temporal genetic variance to a putative spatial genetic structure.

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