Genetic variation of the razor clam *Ensis siliqua* (Jeffreys, 1875) along the European coast based on random amplified polymorphic DNA markers

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Abstract

Ensis siliqua is regarded as an increasingly valuable fishery resource with potential for commercial aquaculture in many European countries. The genetic variation of this razor clam was analysed by randomly amplified polymorphic DNA (RAPD) in six populations from Spain, Portugal and Ireland. Out of the 40 primers tested, five were chosen to assess genetic variation. A total of 61 RAPD loci were developed ranging in size from 400 to 2000 bp. The percentages of polymorphic loci, the allele effective number and the genetic diversity were comparable among populations, and demonstrated a high level of genetic variability. The values of Nei's genetic distance were small among the Spanish and Portuguese populations (0.051– 0.065), and high between these and the Irish populations. Cluster and principal coordinate analyses supported these findings. A mantel test performed between geographic and genetic distance matrices showed a significant correlation (r=0.84, P<0.05), suggesting an isolation by distance process.

Keywords: *Ensis siliqua*, razor clam, RAPD, genetic variation.

Introduction

The razor clam *Ensis siliqua* (Linnaeus, 1758) belongs to the family Pharidae. Populations of this infaunal marine bivalve inhabit fine sand, silt or sandy-mud bottoms at depths between 3 and 12 m where they form extensive and dense beds (Gaspar, Castro & Monteiro 1999). They are highly adapted for living in these habitats due to their possession of a large and powerful foot, which enables rapid vertical burrowing into

sand to depths up to 60 cm. The characteristic shape of their shell, elongated, laterally compressed, narrowed and with almost semicylindrical valves, facilitates rapid burrowing.

Commercial exploitation is a current concern in some bivalve fisheries because exploitation rates are not sustainable. Despite the development of the razor clam's market in recent years attaining high values, the species are not yet under severe exploitation on a wide geographic basis. Nevertheless, the importation rates of this mollusc group are quite significant in Europe, representing a total value of 550 million euros for the year 2004, with Spain importing 47% of this value (according to data obtained from Eurostat information database). Only two genera of razor clams are commercially exploited in Europe, *Ensis* and *Solen*; *E. siliqua* as one of the most widespread species in European markets, especially in the canning sector.

Despite interest from the fishing industry in razor clam aquaculture, little is known about its growth and reproduction. *Ensis siliqua* has an annual reproductive cycle, with only one spawning period during spring and summer (Gaspar & Monteiro 1998; Darriba, San Juan & Guerra 2005). Moreover, to date there is no information available on the extent of molecular genetic structure and genetic variability in different populations of *E. siliqua*. This information is essential for the development of sustainable aquaculture of this species, to ensure appropriate broodstock management programmes and effective breeding programmes to avoid significant reduction in genetic diversity of commercially exploited stocks (Klinbunga, Khamnamtong, Puanglarp, Tassanakajon, Jarayabhand, Hirono, Aoki & Menasveta 2003). In addition, understanding the genetic diversity of the species in a biogeographical context would enable identification and protection of genetically distinct populations (Hassanien, Elnady, Obeida & Itriby 2004).

The use of suitable molecular genetic markers has provided a new horizon for the characterization of the population genetic structure of many taxa. Random amplified polymorphic DNA (RAPD) analysis is a technique based on polymerase chain reaction (PCR) using short oligonucleotides of arbitrary sequence for amplification of discrete regions of the genome (Welsh & McClelland 1990; Williams, Kubelik, Livak, Rafalski & Tingey 1990). The main advantages of the RAPD technique are that it allows the detection of multiple loci, does not require prior knowledge of the genome of the organism in question and is very easy to perform. Random amplified polymorphic DNA analysis has been extensively used to detect genetic diversity and to evaluate the population structure in molluscs (e.g. Huang, Peakall & Hanna 2000; Holmes, Witbaard & van der Meer 2003; Toro, Ojeda & Vergara 2004; Casu, Maltagliati, Cossu, Lai, Galletti, Castelli & Commito 2005; Barreiro, Couceiro, Quintela & Ruiz 2006; Homes & Miller 2006).

The aim of the present study was to characterize novel RAPD markers to analyse the genetic variability and population structure of *E. siliqua* species along the European coast.

Material and methods

<u>Population sampling</u>

Samples of razor clams were collected by dredging or by diving at six localities: three from Spain: Celeiro (43°43′N/7°35′O), Fisterra (42°55′N/9°14′O) and Barra (42°15′N/8°46′O); two from Portugal: Setúbal (38°01′N/8°53′O) and Olhão (37°01′N/7°50′O); and one from Ireland: Strangford Lough (54°29′N/5°36′O) (Fig. 1). About 50 razor clams (ranging in size from 12 to 15 cm) from each locality were delivered alive to the laboratory where they were immediately dissected, and a piece of foot was stored in 95% ethanol for subsequent DNA extraction.
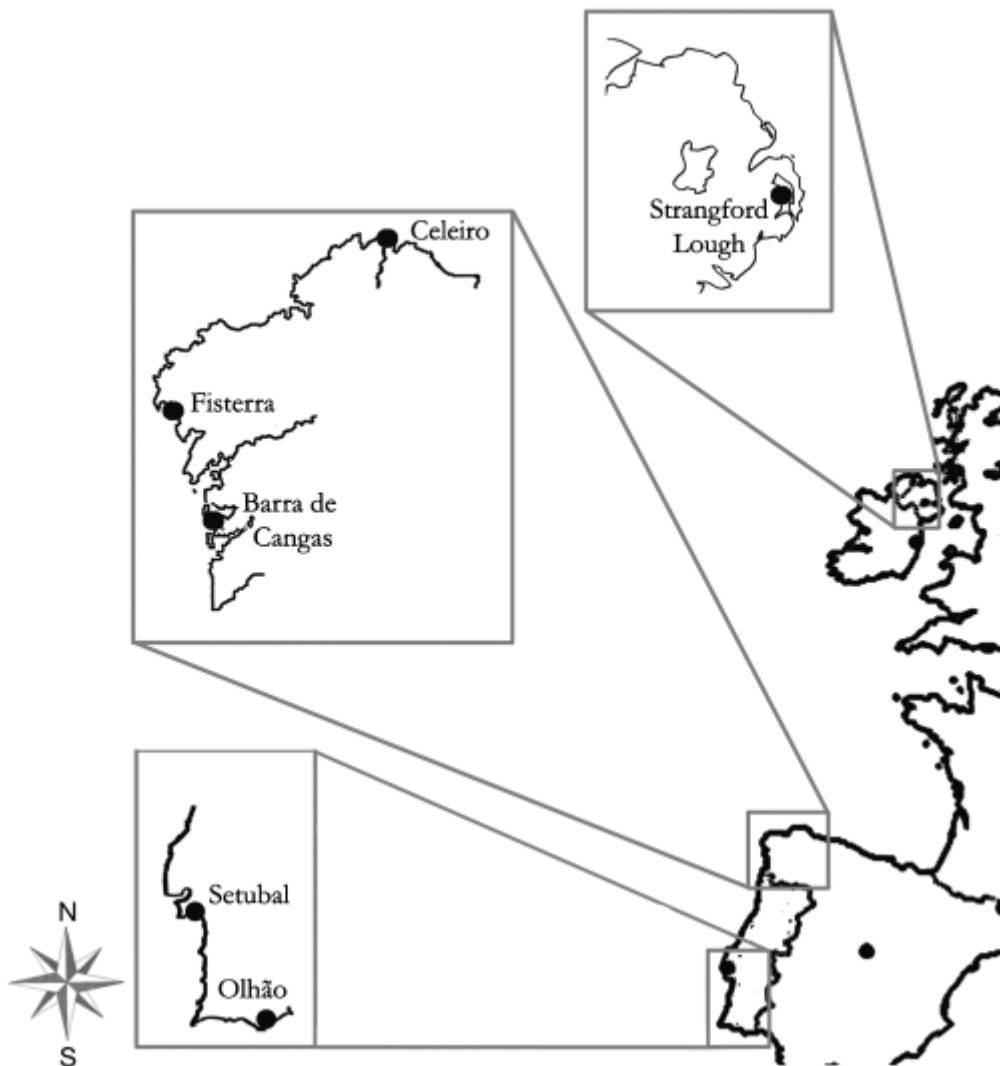
Figure 1. Map showing sampling locations of six populations of *Ensis siliqua* along the European coast.

DNA extraction and amplification analysis

Approximately 50–75 mg of each freshly fixed foot tissue was chopped and digested in 450 µL of lysis buffer (1 M Tris, 0.2 M EDTA, 2.5% N-Lauryl-sarcosine) containing 10 µL of proteinase K (2 mg mL^{-1}) (Sigma-Aldrich, Madrid, Spain), mixed by vortex and incubated at 60°C overnight. A volume of 200 µL of ammonium acetate (3 M, pH 6.0) was added followed by isopropanol precipitation. The extracted DNA was resuspended in 100 µL of distilled water. DNA quality was determined by horizontal electrophoresis in 1% agarose gels with 1 × TAE buffer (40 mM Tris, 2 mM EDTA, 20 mM acetic acid, pH 8.4) and quantified using a Life Science UV/Vis DU 530 spectrophotometer (Beckman Coulter, Madrid, Spain). Random amplified polymorphic DNA using the PCR was carried out in 25 µL of reaction volume containing 25 ng genomic DNA, 1.25 U *Taq* DNA polymerase, 0.2 mM dNTPs, 0.2 µM of each tested primer and 1.75 mM MgCl$_2$. A total of 40 primers of arbitrary sequence and 10 bp in length were used. The PCR reaction was carried out in an MJC-Thermocycler (Bio-Rad Laboratories S.A., Madrid, Spain) with the following programme: an initial denaturation cycle of 3 min at 94°C, followed by 10 low-astringency cycles at 94°C for 45 s, 40°C for 1 min and 72°C for 75 s, then, 30 high-astringency cycles at 94°C for 45 s, 50°C for 1 min and 72°C for 1 min. A final extension was carried out at 72°C for 5 min. A negative control with template DNA replaced by water was performed for each set of amplifications in order to verify the absence of contamination. To ensure reproducibility, RAPD patterns were tested for the

effect of Mg^{2+}, DNA, dNTP and *Taq* DNA polymerase concentrations independently, which can affect the size and the number of amplified products. Also, PCR for each DNA sample was performed at least twice. Polymerase chain reaction products were resolved using agarose gel (1.5%) electrophoresis in $1 \times$ TAE. Two lines of 100 bp molecular ladder (Roche Diagnostics, Barcelona, Spain) were used to estimate and score the size of the PCR fragments. Gels were stained by immersion in 0.5 µg mL^{-1} ethidium bromide for 30 min, visualized on a transilluminator and the image recorded with a UVP (Ultra-Violet Products, Gel Documentation System ImageStore 5000, v7.12, Ultra-Violet Products Ltd, Cambridge, UK) video camera. Molecular size of the RAPD fragments was estimated using the program gelworks (Gelwld 2.51, Ultra-Violet Products Ltd, Cambridge, UK).

RAPD analysis

The number of bands >200 bp but <2000 bp produced for each sample for each primer was then scored manually for presence (1) or absence (0). The differences in band intensities were not taken into account to avoid errors introduced by competition among priming sites during the initial rounds of PCR (Bachmann 1997). The data matrix was entered into the program POPGENE (Yeh, Yang, Boyle, Ye & Mao 1997) with the following genetic parameters calculated: the percentage of polymorphic bands (PPB), number of effective alleles (n_e) and Nei's (1987) gene diversity (h). Nei's genetic distance (Nei 1978), PhiPT (analogous of F_{ST} fixation index) and gene flow were calculated by means of GENALEX software (Peakall & Smouse 2006). A Mantel test between a geographic and genetic distance matrix was performed using the Mantel routine in GENALEX software using 9999 randomizations. In addition, the matrix of Nei's genetic distance was used to cluster the populations by the unweighted pair group method with arithmetic average using UPGMA routine implemented in the tools for population genetics analysis ver. 1.3 software (Miller 1997). The robustness of the dendogram was tested using 1000 bootstrapping. A principal coordinate analysis (PCA) was conducted with genalex software (Peakall & Smouse 2006). This multivariate approach was chosen to complement the cluster analysis information, because cluster analysis is more sensitive to closely related individuals whereas PCA is more informative regarding distances among major groups (Hauser & Crovello 1982).

Results

A total of five primers generated an appropriate amplification pattern with clear and reproducible bands (Table 1). The number of RAPD bands produced per primer varied between 13 (ABA-5 primer) and 12 (rest of primers). A total of 61 reproducible bands were scored, with fragments ranging in molecular size from approximately 200 to 2000 bp.

Table 1. Primers selected for RAPD analyses

Primer	Sequence	No of score bands
ABA-05	5′-AGGGGTCTTG-3′	13
ABA-06	5′-GGTCCCTGAC-3′	12
ABA-15	5′-TTCCGAACCC-3′	12
ABA-16	5′-AGCCAGCGAA-3′	12
AB9-06	5′-GGGAACCCGT-3′	12

RAPD, randomly amplified polymorphic DNA.

Number of polymorphic loci, effective allele number and Nei's genetic diversity for each population are shown in Table 2. The percentage of polymorphic loci ranged between 91.80% for the population of Celeiro and 98.36% for the populations of Fisterra, Barra and Strangford Lough. The number of effective alleles per locus ranged from 1.521 to 1.625 among the six populations of *E. siliqua*. The values of genetic diversity did not vary considerably between populations, ranging from 0.307 to 0.361. The effective number of alleles per locus and Nei's genetic diversity indicated that Portuguese populations exhibited the lowest levels of variability (Table 2).

Table 2. Genetic variability at six *Ensis siliqua* populations detected by RAPD

Population	PPB (%)	n_e	h
Galicia			
Fisterra	98.36	1.625	0.361
Celeiro	91.80	1.548	0.320
Barra	98.36	1.622	0.356
Portugal			
Setúbal	93.44	1.524	0.308
Olhão	96.72	1.521	0.307
Ireland			
Strangford Lough	98.36	1.544	0.323
Total mean	96.17	1.564	0.329

PPB, **percentage**: percentage of polymorphic loci; n_e, effective number of alleles per locus; h, Nei's genetic diversity (Nei 1978).

Nei estimator (Nei 1978) was used to calculate genetic distances, *D*, among the different populations (Table 3). The values obtained ranged from 0.051 to 0.224. In general, the highest distant values were those obtained between the Strangford Lough population and the rest of populations studied. On the other hand, Galician populations showed similar values, ranging from 0.051 to 0.065, having a smaller genetic distance among them. To investigate a possible correlation between genetic relationships and geographic distances, we compared Nei's genetic distance matrix with a corresponding geographic matrix by means of a Mantel test (Table 3). The two matrices showed a highly significant correlation (r=0.834, P=0.010).

Table 3. Genetic distance and geographic distance in six populations of *Ensis siliqua*

Population	Fisterra	Celeiro	Barra	Setúbal	Olhão	Strangford Lough
Fisterra	–	164	194	596	751	1199
Celeiro	0.054	–	81	495	671	1309
Barra	0.065	0.051	–	420	593	1376
Setúbal	0.139	0.102	0.123	–	192	1793
Olhão	0.163	0.169	0.118	0.160	–	1948
Strangford Lough	0.174	0.190	0.214	0.224	0.188	–

Note: Geographic distance (km) above diagonal, Nei's genetic distance below diagonal (Nei 1978).

The fixation index values of PhiPT were close to zero in Galician population pairs (0.051–0.069) and different from zero for the rest of the population pairs, ranging from 0.126 to 0.241 (Table 4). The highest

values were obtained between Portuguese and Irish locations. To estimate the genetic flow among the different populations, the effective migrants number by generation, N_m (Wright 1951), was calculated on the basis of PhiPT values. Based on the number of migrants, the highest gene flow was observed among Galician populations (from N_m=3.357 to 4.637) and the lowest between the Irish population and all other populations (ranging from N_m=0.787 to 0.995) (Table 4).

Table 4. PhiPT values and number of migrants in six populations of *Ensis siliqua* based on 61 RAPD loci

Population	Fisterra	Celeiro	Barra	Setúbal	Olhão	Strangford Lough
Fisterra	–	4.517	3.357	1.308	1.158	0.995
Celeiro	0.052	–	4.637	1.736	1.027	0.945
Barra	0.069	0.051	–	1.425	1.572	0.859
Setúbal	0.160	0.126	0.149	–	1.069	0.787
Olhão	0.178	0.196	0.137	0.190	–	0.986
Strangford Lough	0.184	0.209	0.225	0.241	0.202	–

Number of migrants above diagonal, PhiPT values below diagonal.

The UPGMA phenogram based on Nei's genetic distance matrix is shown in Fig. 2. The Strangford Lough population is the most distinct, while Galician populations form a unique cluster. Regarding Portuguese populations, it should be mentioned that the Olhão population is separated from the other Portuguese populations. Genetic similarities among the 173 individuals were also examined by PCA. The PCA method supported the previous results, clearly separating the Strangford Lough population from the other razor clam populations analysed (Fig. 3). The first three coordinates explained 61.48% of the total variation.
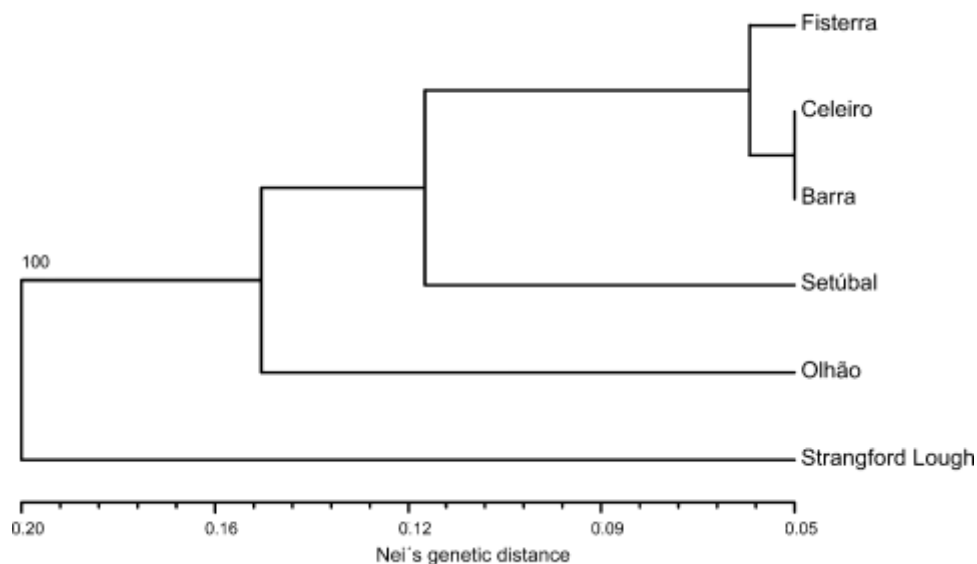

Figure 2. Nei's genetic distance UPGMA topology, based on all 61 random amplified polymorphic DNA loci. Bootstrap values greater than 50% are shown above the nodes.
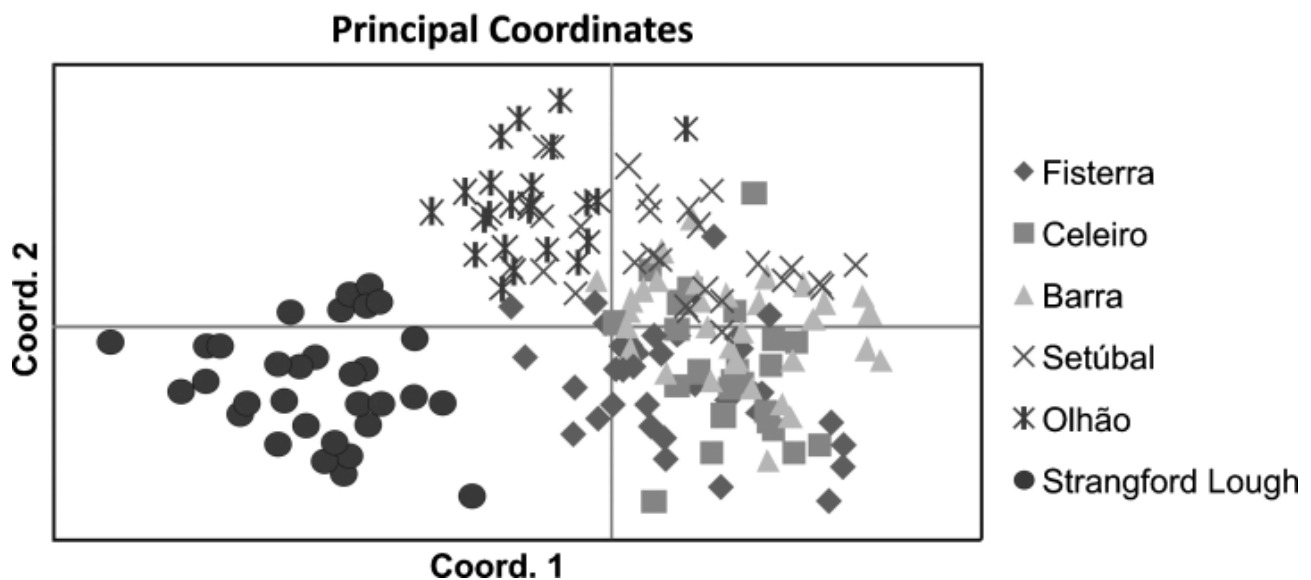
Figure 3. Principal coordinate analysis of six *Ensis siliqua* populations based on 61 random amplified polymorphic DNA loci. Note the separation between the Strangford Lough population and the rest of the populations.

Discussion

Despite the importance of *E. siliqua* as a highly valuable seafood, information on the genetic background of natural populations is not available. The results of the present investigation constitute the first report on the use of RAPD markers to study genetic variation in the razor clam *E. siliqua* and demonstrate the usefulness of RAPD analyses to reveal the genetic differentiation among populations. The RAPD technique has previously been successfully employed in the population analysis of a number of other bivalve species (e.g. Patwary, Kenchington, Bird & Zouros 1994; Heipel, Bishop, Brand & Thorpe 1998; Star, Apte & Gardner 2003; Holmes, Dekker & Williams 2004; Toro et al. 2004; Holmes & Miller 2006).

Analysis of RAPD-amplified fragments from 173 individuals of six populations of *E. siliqua* along the Atlantic East European coast revealed a high genetic variability and a low genetic differentiation among populations. The genetic variability within populations was estimated by means of the number of polymorphic loci (PPB), by effective number of alleles per locus (n_e) and by Nei's genetic diversity (h). The results ranged from 91.80% to 98.36% for PPB, from 1.521 to 1.625 for n_e and from 0.307 to 0.361 for h. These results are slightly higher than in other bivalves as in populations of the mussel *Mytilus galloprovincialis* in Southern California (Li Ma, Cowles & Carter 2000), or in several populations of the gasteropod *Littorina striata* (de Wolf, Backeljau & Verhagen 1998). The genetic variability within populations is a very important measure of species adaptation to environmental changes and, therefore, of species survival (Sofia, Silva, Galindo, Almeida, Sodré & Cláudia 2006). When the gene pool of a population narrows and loses genetic plasticity, it becomes increasingly susceptible to changes in the environmental conditions and hence more prone to extinction (Guttman & Berg 1998).

The high values of variability within populations and the low levels of genetic variation among populations suggest the existence of a reproduction model without inbreeding and panmixy. Such a model would require a high heterozygosity within populations and a low genetic variability between populations, because of a high gene flow due to the lack of geographic barriers (Armbruster 1997, 1998). However, the population sampled in Strangford Lough was genetically distinct from the others, although the scale of differentiation was not too large (0.174–0.224) (Table 3). A possible explanation for this observation lies in the isolation caused by the location and hydrological features of Strangford Lough. The lough is situated in the Irish Sea to which it is connected by a narrow channel; this may limit movement of larvae outside the embayment. Water circulation within the lough may also further entrain locally produced larvae as suggested by Kennedy and Roberts (2006). The low genetic distance between Galician populations indicates that they act as a single

interbreeding population, possibly with high levels of gene flow between them. Therefore, it would be more appropriate to call them localities instead of populations. At the same time, the Mantel test performed showed a significant correlation (r=0.834, P<0.010) between geographic and genetic distance. This seems to be an effect of the geographic distances on genetic variation, which is in accordance with an isolation-by-distance process, defined by Slatkin (1993) as a decrease in genetic similarity among populations with increasing geographic distance between them.

PhiPT and F_{ST} are analogous standardizing measures of the degree of genetic differentiation among populations; scores for both measures range from 0 (no differentiation) to 1 (no alleles shared). According to Wright (1978), values of F_{ST} ranging from 0.05 to 0.15 are indicative of moderate population genetic structuring, while values from 0.15 to 0.25 indicate high genetic structuring. The PhiPT values obtained for *E. siliqua* suggest low differentiation among Galician populations, a moderate differentiation between Galician and Portuguese locations and a higher differentiation between Strangford Lough and each of the other sites (Table 4). These results reflect a pattern that may be caused by an isolation-by-distance process.

Gene flow is the most important determinant of the genetic structure of populations, since it controls to what extent each local population of a species is an independent evolutionary unit (Slatkin 1993). If gene flow among nearby populations is intense, they evolve together, while if the gene flow is small, each population evolves independently. Theoretically, a value of N_m>1 is necessary to prevent random differentiation by genetic drift (Slatkin 1987). Thus, the number of migrants among Galician populations reported in this study (N_m=3.357–4.637) suggests that the gamete exchange is responsible for avoiding genetic differentiation between them, and supports the previous idea that they act as a single interbreeding population. The number of migrants between Portuguese and Galician populations was higher than 1 in all comparisons buffering the effects of the stochastic processes of drift. However, the results exhibited by Strangford Lough (N_m<1 in all comparisons) reflect that the gene flow is not enough to avoid differentiation by genetic drift. Nevertheless, the estimates of gene flow extrapolated from F_{ST} or analogous statistics must be taken into account cautiously because the mathematical assumptions underlying estimates of genetic differentiation may not be totally realistic (Whitlock & McCauley 1999). The data of N_m obtained by indirect methods reflect the historic average of gene flow needed to explain the differentiation model observed among populations, and this overestimate the real gene flow (Slatkin 1985). Nevertheless, many authors have estimated the number of effective immigrants per generation to infer patterns of gene flow from FST using RAPDs in gasteropod molluscs (de Wolf et al. 1998), insects (Apostol, Black, Reiter & Miller 1996; Zhou, Faktor, Applebaum & Coll 2000) and fish (Mamuris, Stamatis & Triantaphyllidis 1999).

Phenotypic analyses by means of the UPGMA method and PCA support the previous findings of low genetic variation among Galician populations and the distinctiveness of the Strangford Lough population. The main cluster in the UPGMA tree with Galician and Portuguese populations (Fig. 2) suggests that larval transport along the coast occurs over long distances. It should be noted that Olhão is separated from Setúbal, which is closer to Galician populations, thus supporting the idea of an effect of geographic distance over genetic distance.

The use of RAPDs has been shown to be appropriate for the study of the genetic structure of different populations of *E. siliqua*. The results from the present study are consistent with the life-history features of the razor clam *E. siliqua* with a prolonged larval pelagic life span (up to 1 month) (Martínez-Patiño 2002), which suggest a great potential for genetic homogenization over large geographical distances. The discovery of moderate genetic differentiation between Galician and Portuguese populations and the high differentiation between these and the Irish population is relevant to the management of this marine resource. To avoid genetic bottle-neck effects or the introduction of stock that is genetically distinct from local populations, genetic information should be used to inform the design of restocking strategies, particularly in populations such as those from Portugal, which have suffered strong declines due to overfishing (Gaspar et al. 1999). In addition, local geographical and hydrological features, such as those in Strangford Lough, could exacerbate

isolation and play a major role in the genetic structuring of different populations. In this context, it is important to improve our understanding of the relative contribution of distance and local hydrographical features to genetic differentiation among *E. siliqua* populations by analysing additional populations using the techniques developed in the present study.

Acknowledgments

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