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ORIGINAL ARTICLE

Genetic analysis of two Portuguese populations of *Ruditapes decussatus* by RAPD profiling

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Abstract The clam *Ruditapes decussatus* is commercially important in the south of Portugal. The random amplified polymorphic DNA (RAPD) technique was applied to assess the genetic diversity and population structure of two Portuguese populations occurring in the Ria Formosa (Faro) and the Ria de Alvor, respectively. Twenty-five individuals of each population were investigated by RAPD profiles. Genetic diversity within populations, measured by the percentage of polymorphic loci (*%P*), varied between 68.57% (Alvor) and 73.88% (Faro). Shannon's information index (*H*) and Nei's gene diversity (*h*) were 0.281 and 0.176, respectively, for the Alvor population and 0.356 and 0.234 for the Faro population. Overall, genetic variation within *R. decussatus* populations was high. The total genetic diversity (H_T) was explained by a low variation between populations ($G_{ST} = 0.145$), which is consistent with high gene flow ($N_m = 2.9$). The analysis of molecular variance (AMOVA) showed that 65% of variability is within populations and 35% between populations ($\Phi_{PT} = 0.345$; $P \geq 0.001$). The value of Nei's genetic distance was 0.0881, showing a low degree of population genetic distance, despite the different geographic origin. This is the first study on the population genetics of *R. decussatus* by RAPD technique. The results may be useful for restocking programs and aquaculture.

Keywords RAPDs · *Ruditapes decussatus* · Genetic diversity · Genetic differentiation

Abbreviations

RAPDs	Random amplified polymorphic DNA
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline solution
s	Seconds
min	Minutes
dNTPs	Deoxyribonucleotide triphosphate
bp	Base pair

Introduction

The grooved carpet shell clam, *Ruditapes decussatus*, is widely distributed along the coastal and estuarine areas of Europe and North Africa. In Portugal, *R. decussatus* is among the most common clam species found in the market for human consumption and the one with the highest economic importance. Its market price can reach four times the price of other clam species (Fernández et al. 2000). About 80% of shellfish production originates from aquaculture, which is based on seed collected from natural recruitment

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banks and planted by producers in ongrowing parks (Matias et al. 2009). In spite of the species' great commercial importance, studies on the genetic diversity are very scarce, regarding only to allozymes (Worms and Pasteur 1982; Jarne et al. 1988; Borsa et al. 1991; Borsa et al. 1992; Jordaens et al. 2000). A sustainable exploitation of the natural resource and improvement in aquaculture requires a deeper knowledge of the genetic variability and genetic relationships of the different clam populations.

The classical strategies for the evaluation of genetic variation, such as morphology and embryology, have been outdated by the availability of molecular markers techniques (Weising et al. 1995). In general, molecular markers are based on polymorphisms found in DNA. The values obtained will be different for each individual, population or species, depending of the resolution of the DNA markers used. One of the molecular markers technique currently used is the random amplified polymorphic DNA (RAPD) (Williams et al. 1990; Welsh and McClelland 1990) that provides a useful tool to study the genetic variability of a population. This technique consists in the PCR (polymerase chain reaction) amplification of small, inverted repeats scattered in the genome, using a single, short primer of arbitrary sequence, allowing a scan throughout the genome, more randomly than the conventional techniques. The ability to examine genomic variation without previous sequence information (Williams et al. 1990), the relatively low cost of the technique and the requirement of only some nanograms of template DNA represent the major advantages of the use of RAPD technique in population studies. RAPDs have showed to be an extremely sensitive method for detecting DNA variation and for the establishment of genetic relationships between closely related organisms (Péres et al. 1998). Among the multiple applications of RAPDs are their use in population genetic studies (Haig et al. 1994), taxonomy (Chapco et al. 1992), determination of paternity (Lewis and Snow 1992) and mapping (Michelmore et al. 1991). The two main and often mentioned disadvantages of this type of molecular markers are a lack of reproducibility and the loss of complete genotypic information, due to the fact that most RAPD bands are dominantly inherited. The potential use of RAPD analysis in phylogenetic studies and population genetics has been documented in a wide variety of organisms (Hadrys et al. 1992). In marine invertebrates, this technique has successfully been used in analysing genetic polymorphisms at the interspecific level in oysters (Klinbunga et al. 2000) and mussels (Rego et al. 2002) and at the intraspecific level in scallops (Patwary et al. 1994), abalone (Huang et al. 2000) and oysters (Hirschfeld et al. 1999; Klinbunga et al. 2001).

In the present study, RAPD profiling was applied to measure the genetic diversity and interpopulational differentiation of two Portuguese populations of the clam *R. decussatus*.

Materials and methods

Sample collection and DNA extraction

Ruditapes decussatus individuals were collected from two Portuguese populations, 25 individuals from Ria Formosa (Faro: 37° 2′ 0″ N, 7° 55′ 0″ W) and 25 individuals from Ria de Alvor (Alvor: 37° 8′ 0″ N, 8° 36′ 0″ W), Algarve, south of Portugal. After two days of depuration, the material was dissected and kept in 70% ethanol, until further use. The protocol for DNA extraction followed the one by Pereira (2008), where 5–25 mg of adductor muscle was placed in a 2-ml eppendorf, with 100 µl of 1 × PBS and distilled water for 10 min each. The tissue was cut in small pieces and homogenized with a Pestle Pellet (Sigma). Afterwards, 180 µl MDT (Tissue Lysis Buffer) and 20 µl EDT (Proteinase K) were added and the samples were incubated overnight at 55°C for complete digestion. After centrifugation at 10000 rpm, for 3 min, at room temperature, the supernatant was transferred to a new 2-ml eppendorf. The subsequent addition of 180 µl LDT (Lysis Buffer) was followed by a 15-s vortexing, and a flash spins down. After incubation at 70° C for 10 min, 240 µl of absolute ethanol was added and the tubes were vortexed and spinned down. Finally, the lysate was transferred to a cartridge of the automatic nucleic-acid isolation system QuickGene-800, and the "DNA tissue mode" was selected.

PCR amplification

Several experiments were made to optimize the reproducibility of the RAPD assay, closely following the protocol of Williams et al. (1990), in which concentrations of template DNA, dNTPs, $MgCl_2$ concentration and *Taq polymerase* were varied to determine which conditions produced the strongest and most reproducible patterns. Amplification reactions were performed in volumes of 25 µl containing 50 ng of genomic template DNA, 100 µM of each dATP, dCTP, dGTP and dTTP, 0.2 µM of the primer and 0.5 units of Taq DNA polymerase (Fermentas, Life Sciences). Amplification was performed in a Thermal Cycler (T-personal, Biometra) in a total of 45 cycles: 1 min at 94°C, 1 min at 30–36°C and 2 min at 72°C. The total volume of the PCR products was evaluated in 2% agarose gels and visualized by ethidium bromide staining. After electrophoresis, DNA bands profiling was observed under UV light and the images were saved in a gel analyser (UVIDOC).

Data analysis

The genetic diversity within populations and the genetic differentiation between populations were quantified using GeneAlEx 6.1, Genetic Analysis Software (Peakall and Smouse 2006) and POPGENE (Yeh et al. 1997) programs. The genetic diversity within populations was estimated by calculating percentage polymorphic loci (*%P*), number of expected alleles (n_a), effective allele number (n_e), Nei's gene diversity (Nei 1973) (*h*), Shannon's information index (*H*), total gene diversity (H_T) and average gene diversity within populations (H_S).

In order to estimate the genetic differentiation between populations, analogues of F_{ST} fixation index measures such as coefficient of gene differentiation (G_{ST}) (Nei 1987) and Φ_{PT} (AMOVA) were used. The analysis of molecular variance (AMOVA) was calculated with the software GeneAlEx 6.1 (Peakall and Smouse 2006) for variation among individuals within populations and between populations. Due to the absence of assumptions, AMOVA is widely applicable and powerful. The fact that allele frequencies are not calculated led to the application of this method to analyse dominant data type, like RAPDs (Huff et al. 1983). The values of gene flow (N_m) (McDonald and McDermott 1993) and standard genetic distance of Nei (1972) were estimated.

Results

Genetic diversity within populations

The application of RAPD technique to the genome of *Ruditapes decussatus* produces a significant number of amplified fragments. Of the twenty screened primers, ten amplified 245 clear and reproducible bands (Table 1). One hundred and sixty-eight bands were amplified in the Alvor population and 183 in the Faro population (Fig. 1), with lengths ranging from 180 to 1,500 bp. The primer OPE-15 generated the highest number of bands (21 bands in the Alvor population and 24 in the Faro population); primer OPE-02 in the Faro population and primers OPE-01 and OPE-16 in the Alvor population produced the lowest number of bands/markers (Table 1). The two populations have 62 (Alvor) and 77 (Faro) private bands, respectively.

A summary of the genetic diversity of the populations based on the RAPD markers is presented in Table 2. Since dominant data have some shortcomings in the calculation of genetic diversity (Lowe et al. 2004), the method developed by Lynch and Milligan (1994) present in GeneAlEx 6.1 Genetic Analysis Software (Peakall and Smouse 2006) was used to calculate allele frequencies. The percentage of polymorphism (*%P*) of the two Portuguese populations was 68.57% (Alvor) and 73.88% (Faro), while the number of expected alleles (n_a) and the number of effective alleles (n_e) were 1.371 and 1.267 for the Alvor and 1.486 and 1.389 for the Faro population. Nei's gene diversity index (*h*) and Shannon's information index (*H*) were estimated for the two populations of *R. decussatus*: the Alvor population showed the values of 0.176 and 0.281, while the values for the Faro population were 0.234 and 0.356.

Population genetic structure

Genetic diversity based on all bands showed that the total gene diversity (H_T), measured in terms of the total expected heterozygosity, was 0.2396, the gene diversity within populations (H_S) was 0.2047, and gene diversity between populations ($D_{ST} = H_T - H_S$) was 0.0349 (Table 3). Using these diversity indices, it was possible to calculate the coefficient of gene differentiation ($G_{ST} = D_{ST}/H_T = 0.1450$), which measures the proportion of total genetic diversity occurring between populations and is analogous to

Table 1 Selected primers for the evaluation of genetic diversity in the two populations of *Ruditapes decussatus*

Name	Sequence (5′–3′)	*T* (°C)	Alvor		Faro	
			Number of bands	Range of bands	Number of bands	Range of bands
OPE-01	CCCAAGGTCC	33	12	250–900	20	380–1,500
OPE-02	GGTGCGGGAA	36	15	300–880	10	520–1,200
OPE-03	CCAGATGCAC	36	17	300–880	16	350–1,100
OPE-06	AAGACCCCTC	36	22	250–1,500	17	250–1,100
OPE-10	CACCAGGTGA	36	17	250–1,300	23	250–1,500
OPE-11	GAGTCTCAGG	36	17	180–1,200	18	180–1,300
OPE-12	TTATCGCCCC	33	15	280–750	12	380–950
OPE-14	TGCGGCTGAG	33	20	320–1,000	21	250–1,000
OPE-15	ACGCACAACC	33	21	200–800	24	180–1,000
OPE-16	GGTGACTGTG	36	12	320–850	21	200–1,500

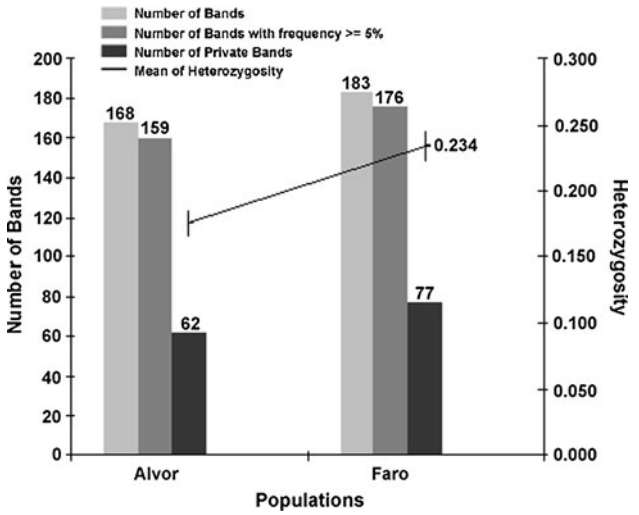

Fig. 1 Number of bands and mean heterozygosity in two *Ruditapes decussatus* populations

Table 2 Genetic variation within two Portuguese populations of *Ruditapes decussatus*

	N		n_a	n_e	*h*	*H*	*%P*
Alvor	25	Mean	1.371	1.267	0.176	0.281	68.57
		SE	0.059	0.018	0.010	0.1654	
Faro	25	Mean	1.486	1.389	0.234	0.356	73.88
		SE	0.056	0.023	0.012	0.017	
Total	50	Mean	1.429	1.328	0.205	0.319	71.22
		SE	0.041	0.015	0.008	0.011	

N number of sampled individuals, n_a number of expected alleles, n_e effective allele number, *h* Nei's gene diversity, *H* mean Shannon's information index; *%P* percentage polymorphic loci calculated with all alleles included, *SE* standard error

Wright's F_{ST} values. This means that only approximately 14.5% of the genetic variation observed in this study was due to differentiation between populations, while 85.5% is due to variation within populations,

Estimates of G_{ST} also allow to estimate another genetic parameter, the gene flow $[N_m = 0.5(1-G_{ST})/G_{ST}]$ (McDonald and McDermott 1993), which was calculated to $N_m = 2.9$. According to Crow and Aoki (1984), gene flow values of $N_m < 1$ should be interpreted as little or no gene flow. Thus, the value of $N_m = 2.9$ would suggest high genetic exchange between the two populations studied.

AMOVA (analysis of molecular variance) (Table 4) showed that 65% of the variability was within populations whilst 35% was between them. Analogue to Wright's F_{ST} is also the Φ_{PT} value, estimated with AMOVA; it amounted to 0.345 ($P \geq 0.001$), based on permutation across the full data set.

Table 3 Genetic variation between the Portuguese populations of *Ruditapes decussatus*, based on the diversity indices of Nei (1973)

H_T	Total gene diversity	0.2396
H_S	Gene diversity within populations	0.2047
D_{ST}	Gene diversity between populations	0.0349
G_{ST}	Coefficient of gene differentiation	0.1450
N_m	Gene flow	2.9

Table 4 Analysis of molecular variance (AMOVA) in the two Portuguese populations of *Ruditapes decussatus*

Source	*df*	SS	Variance (%)	Fixation index (Φ_{PT})
Between pops	1	463.320	35	0.345
Within pops	48	1569.520	65	
Total	49	2032.840	100	

To compare the degree of genetic differentiation between populations, the standard genetic distance of Nei (1972) was estimated. The value of Nei's genetic distance was $D = 0.0881$, while the value of genetic identity was $I = 0.9157$, indicating low genetic differentiation between the two populations.

Discussion

RAPD assay was used in order to analyse the genetic diversity and differentiation in two southern Portuguese populations of *Ruditapes decussatus*. The technique was found to be effective in revealing polymorphisms in this species, since all 10 random primers produced at least one polymorphic fragment. The major limitations of this technique are its lack of reproducibility (Ford-Lloyd and Painting 1996; Weising et al. 1995; Guadagnuolo et al. 2001) and its sensitivity to small variations in PCR conditions. In order to overcome these limitations, DNA of high quality was used and three repetitions were performed for the same primers and individuals which generate the same results.

Genetic diversity within populations is of great concern to ecologists and geneticists. A reduced genetic variation is thought to affect the ability of populations to adapt to changing environments, thereby increasing their probability of extinction (Beardmore 1983). Waugh and Powell (1992) stated that the analysis of polymorphism by molecular markers could help to select priority areas for conservation and provide vital information for the development of genetic sampling and improvement.

In total, the analysis of the genetic variability within populations by RAPDs based on percentage of polymorphic loci (*%P*), effective allele numbers (n_a), expected numbers of alleles (n_e), Nei's gene diversity (*h*) and Shannon's Index (*H*) suggests that the two populations present very high levels of genetic diversity. These results are similar to those by Borsa and Thiriot-Quievreux (1990), Borsa et al. (1992), Passamonti et al. (1997) and Jordaens et al. (2000) who studied the populations of this species, especially the Azores population, using allozymes as molecular markers. The high levels of genetic variation suggest that these bivalves are phenotypically plastic and may respond adequately to changing environments. In contrast, very low genetic differentiation was found between the two studied populations. Only 14.5% ($G_{ST} = 0.145$) of the genetic variability was between populations, while 85.5% was within populations. The gene flow (N_m) value (Slatkin 1993) allows evaluating whether or not each of the considered population of a species evolves as an independent unit. Theory predicts that a global value of $N_m > 1$ prevents random differentiation by genetic drift (Slatkin 1993). The calculated level of gene flow ($N_m = 2.9$) would allow the two populations to act as a single interbreeding population. However, this conclusion should be considered cautiously because the mathematical assumptions underlying the estimates of genetic diversity and differentiation may not be completely realistic (Whitlock and McCauley 1999). Similar results were obtained with the method of analysis of molecular variance (AMOVA) that showed a high genetic variation within populations and low genetic differentiation between them. However, the value of fixation index ($\Phi_{PT} = 0.345$) suggested that the populations may have some structure, although the degree of differentiation was not large. This differentiation may be due to geographical isolation or hydrological factors.

The values of the distance and genetic identity (Nei 1972) estimated for the two populations were 0.9176 and 0.0860, respectively. These values support the high degree of genetic identity and the low level of genetic differentiation of the two populations.

In conclusion, the two populations of *R. decussatus* studied the presented high values of genetic variability within populations and low values of genetic differentiation between them, demonstrating a high degree of genetic homogeneity. The value of the gene flow (N_m), G_{ST} and Φ_{PT} quantification demonstrates that the exchange of genes between the two populations is high. A high degree of genetic homogeneity among populations has also been described for the populations of other marine invertebrates, such as *Concholepas concholepas* (Gallardo and Carrasco 1996), *Ostrea chilensis* (Toro and Aguila 1996) and *Venus antique* (Gallardo et al. 1998). The high values found for the genetic diversity within populations, associated with low values of genetic variation between populations, suggest panmixy as the dominant mode of reproduction (Armbruster 1997, 1998; Fernández-Tajes et al. 2007).

Mollusc species with extended larval planktonic phases are generally thought to disperse further and to show higher rates of gene flow, larger geographic ranges, lower levels of genetic differentiation between populations and high levels of genetic variation within populations (Scheltema and Williams 1983; Waples 1987; Williams and Benzie 1993; Palumbi 1995). The life cycle of *R. decussatus* presents a larval planktonic stage, which lasts about three weeks, followed by benthic juvenile and adult stages. During the larval phase, larvae can travel many miles driven by currents before settling in favourable habitats.

Due to overexploitation, the *R. decussatus* population at Alvor suffered a strong decline. This led to a restocking by man for commercial purposes with seed from other local populations, mainly from the Ria Formosa population, which is the most important source of *R. decussatus* natural seed (juveniles) for these activities. The dispersal of planktonic larvae as well as the introduction of Ria Formosa seed in the Alvor population may have contributed to the low values of genetic differentiation between these two populations. These results can be very useful in designing programmes for the management of marine resources. Furthermore, studies on the genetic variability of *R. decussatus* should be extended to a larger number of local population using other types of molecular markers in order to provide a broader scientific for defining the best practices to improve a sustainable aquaculture production.

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