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1 **Title: Genetic analysis of two Portuguese populations of**  
2 ***Ruditapes decussatus* by RAPD profiling**

3

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22 **Abstract**

23 The clam *Ruditapes decussatus* is commercially important in the South of Portugal. The Random  
24 Amplified Polymorphic DNA (RAPD) technique was applied to assess the genetic diversity and  
25 population structure of two Portuguese populations occurring in the Ria Formosa (Faro) and the Ria de  
26 Alvor, respectively. Twenty five individuals of each population were investigated by RAPD profiles.  
27 Genetic diversity within populations, measured by percentage of polymorphic loci (%P), varied between  
28 68.57% (Alvor) and 73.88% (Faro). Shannon's information index ( $H$ ) and Nei's gene diversity ( $h$ ) were  
29 0.281 and 0.176, respectively, for the Alvor population, and 0.356 and 0.234 for the Faro population.  
30 Overall, genetic variation within *R. decussatus* populations was high. The total genetic diversity ( $H_T$ ) was  
31 explained by a low variation between populations ( $G_{ST}=0.145$ ), which is consistent with high gene flow  
32 ( $N_m=2.9$ ). The analysis of molecular variance (AMOVA) showed that 65% of variability is within  
33 populations and 35% between populations ( $\Phi_{PT} = 0.345$ ;  $P \geq 0.001$ ). The value of Nei's genetic distance  
34 was 0.0881, showing a low degree of population genetic distance, despite the different geographic origin.  
35 This is the first study on the population genetics of *R. decussatus* by RAPD technique. The results may be  
36 useful for restocking programs and aquaculture.

37 **Keywords:** RAPDs, *Ruditapes decussatus*, Genetic diversity, Genetic differentiation

38 **Abbreviations:** RAPDs, Random Amplified Polymorphic DNA; PCR, Polymerase Chain Reaction; PBS,  
39 Phosphate Buffered Saline Solution; s, seconds; min, minutes; dNTPs, Deoxyribonucleotide triphosphate;  
40 bp, base pair;

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43 **1. Introduction**

44 The grooved carpet shell clam, *Ruditapes decussatus*, is widely distributed along the coastal and  
45 estuarine areas of Europe and North Africa. In Portugal, *R. decussatus* is among the most common clam  
46 species found in the market for human consumption, and the one with the highest economic importance.  
47 Its market price can reach four times the price of other clam species (Fernández et al. 2000). About 80 %  
48 of shellfish production originates from aquaculture, which is based on seed collected from natural  
49 recruitment banks and planted by producers in ongrowing parks (Matias et al. 2009). In spite of the  
50 species' great commercial importance, studies on the genetic diversity are very scarce, regarding only to  
51 allozymes (Worms et al. 1982; Jarne et al. 1988; Borsa et al. 1991; Jordaens et al. 2000). A sustainable  
52 exploitation of the natural resource and improvement in aquaculture requires a deeper knowledge of the  
53 genetic variability and genetic relationships of the different clam populations.

54 The classical strategies for the evaluation of genetic variation, such as morphology and  
55 embryology, have been outdated by the availability of molecular markers techniques (Weising et al.  
56 1995). In general, molecular markers are based on polymorphisms found in DNA. The values obtained  
57 will be different for each individual, population or species, depending of the resolution of the DNA  
58 markers used. One of the molecular markers technique currently used is the Random Amplified  
59 Polymorphic DNA (RAPD) (Williams et al. 1990; Welsh and McClelland 1990) that provides an useful  
60 tool to study the genetic variability of a population. This technique consists in the PCR (Polymerase  
61 Chain Reaction) amplification of small, inverted repeats scattered in the genome, using a single, short  
62 primer of arbitrary sequence, allowing a scan throughout the genome, more randomly than the  
63 conventional techniques. The ability to examine genomic variation without previous sequence  
64 information (Williams et al. 1990), the relatively low cost of the technique and the requirement of only  
65 some nanograms of template DNA, represent the major advantages of the use of RAPD technique in  
66 population studies. RAPDs have showed to be an extremely sensitive method for detecting DNA variation  
67 and for the establishment of genetic relationships between closely related organisms (Péres et al. 1998).  
68 Among the multiple applications of RAPDs are their use in population genetic studies (Haig et al. 1994),  
69 taxonomy (Chapco et al. 1992), determination of paternity (Lewis and Snow 1992) and mapping  
70 (Michelmore et al. 1991). The two main and often mentioned disadvantages of this type of molecular  
71 markers are a lack of reproducibility and the loss of complete genotypic information, due to the fact that  
72 most RAPD bands are dominantly inherited. The potential use of RAPD analysis in phylogenetic studies  
73 and population genetics has been documented in a wide variety of organisms (Hadrys et al. 1992). In  
74 marine invertebrates, this technique has successfully been used in analysing genetic polymorphisms at the  
75 interspecific level in oysters (Klinbunga et al. 2000) and mussels (Rego et al. 2002), and at the  
76 intraspecific level in scallops (Patwary et al. 1994), abalone (Huang et al. 2000) and oysters (Hirschfeld et  
77 al. 1999; Klinbunga et al. 2001).

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

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## 83 2. Material and methods

### 84 2.1. Sample collection and DNA extraction

85 *Ruditapes decussatus* individuals were collected from two Portuguese populations, 25  
86 individuals from Ria Formosa (Faro: 37° 2' 0" N, 7° 55' 0" W) and 25 individuals from Ria de Alvor  
87 (Alvor: 37° 8' 0" N, 8° 36' 0" W), Algarve, South of Portugal. After two days of depuration, the material  
88 was dissected and kept in 70% ethanol, until further use. The protocol for DNA extraction followed the  
89 one by Pereira (2008), where 5-25 mg of adductor muscle was placed in a 2 ml eppendorf, with 100 µl of  
90 1× PBS and distilled water for 10 min each. The tissue was cut in small pieces and homogenized with a  
91 Pestle Pellet (Sigma). Afterwards, 180 µl MDT (Tissue Lysis Buffer), 20 µl EDT (Proteinase K) were  
92 added and the samples were incubated overnight at 55°C for complete digestion. After centrifugation at  
93 10000 rpm, for 3 min, at room temperature, the supernatant was transferred to a new 2 ml eppendorf. The  
94 subsequent addition of 180 µl LDT (Lysis Buffer) was followed by a 15 s vortexing, and a flash spin  
95 down. After incubation at 70° C for 10 min, 240 µl of absolute ethanol was added and the tubes were  
96 vortexed and spun down. Finally, the lysate was transferred to a cartridge of the automatic nucleic-acid  
97 isolation system QuickGene-800 and the "DNA tissue mode" was selected.

### 98 2.2. PCR amplification

99 Several experiments were made to optimize the reproducibility of the RAPD assay, closely  
100 following the protocol of Williams et al. (1990), in which concentrations of template DNA, dNTPs,  
101 MgCl<sub>2</sub> concentration and *Taq polymerase* were varied to determine which conditions produced the  
102 strongest and most reproducible patterns. Amplification reactions were performed in volumes of 25 µl,  
103 containing: 50 ng of genomic template DNA, 100 µM of each dATP, dCTP, dGTP and dTTP, 0.2 µM of  
104 the primer and 0.5 units of Taq DNA polymerase (Fermentas, Life Sciences). Amplification was  
105 performed in a Thermal Cycler (T-personal, Biometra) in a total of 45 cycles: 1 min at 94°C, 1 min at 30-  
106 36°C and 2 min at 72°C. The total volume of the PCR products were evaluated in 2% agarose gels and  
107 visualized by ethidium bromide staining. After electrophoresis, DNA bands profiling were observed  
108 under UV light and the images were saved in a gel analyzer (UVIDOC).

### 109 2.3. Data analysis

110 The genetic diversity within populations and the genetic differentiation between populations  
111 where quantified using: GeneA1Ex 6.1, Genetic Analysis Software (Peakall and Smouse 2007) and  
112 POPGENE 3.2 (Yen et al. 1996) programs. The genetic diversity within populations was estimated by  
113 calculating: percentage polymorphic loci (%P), number of expected alleles ( $n_e$ ), effective allele number  
114 ( $n_e$ ), Nei's gene diversity (Nei, 1973) ( $h$ ), Shannon's information index ( $H$ ), total gene diversity ( $H_T$ ) and  
115 average gene diversity within populations ( $H_S$ ).

116 In order to estimate the genetic differentiation between populations analogous of  $F_{ST}$  fixation  
117 index measures such as coefficient of gene differentiation ( $G_{ST}$ ) (Nei 1987) and  $\Phi_{PT}$  (AMOVA) were  
118 used. The analysis of molecular variance (AMOVA) was calculated with the software GeneA1Ex 6.1  
119 (Peakall and Smouse 2007) for variation among individuals within populations and among populations.  
120 Due to the absence of assumptions, AMOVA is widely applicable and powerful. The fact that allele  
121 frequencies are not calculated, led to the application of this method to analyze dominant data type, like

122 RAPDs (Huff et al. 1993). The values of gene flow ( $N_m$ ) (McDermott and McDonald 1993) and standard  
123 genetic distance of Nei (1972) were estimated.

### 125 3. Results

#### 126 3.1. Genetic diversity within populations

127 The application of RAPD technique to the genome of *Ruditapes decussatus* produces a  
128 significant number of amplified fragments. Of the twenty screened primers, ten amplified 245 clear and  
129 reproducible bands (Table 1). 168 bands were amplified in the Alvor population and 183 in the Faro  
130 population (Fig. 1), with lengths ranging from 180 to 1500 bp. The primer OPE-15 generated the highest  
131 number of bands (21 bands in the Alvor population and 24 in the Faro population); primer OPE-02 in the  
132 Faro population and primers OPE-01 and OPE-16 in the Alvor population, produced the lowest number  
133 of bands/markers (Table 1). The two populations have 62 (Alvor) and 77 (Faro) private bands,  
134 respectively.

135 A summary of the genetic diversity of the populations based on the RAPD markers is presented  
136 in Table 2. Since dominant data have some shortcomings in calculation of genetic diversity (Lowe et al.  
137 2004), the method developed by Lynch and Milligan (1994) present in GeneAEx 6.1 Genetic Analysis  
138 Software (Peakall and Smouse 2007) was used to calculate allele frequencies. The percentage of  
139 polymorphism (%P) of the two Portuguese populations was 68.57% (Alvor) and 73.88% (Faro), while the  
140 number of expected alleles ( $n_a$ ) and the number of effective alleles ( $n_e$ ) were 1.371 and 1.267 for the  
141 Alvor, and 1.486 and 1.389 for the Faro population. Nei's gene diversity index ( $h$ ) and Shannon's  
142 information index ( $H$ ) were estimated for the two populations of *R. decussatus*: the Alvor population  
143 showed values of 0.176 and 0.281, while values for the Faro population were 0.234 and 0.356.

#### 144 3.2. Population genetic structure

145 Genetic diversity based on all bands showed that the total gene diversity ( $H_T$ ), measured in terms  
146 of the total expected heterozygosity, was 0.2396, the gene diversity within populations ( $H_S$ ) was 0.2047,  
147 and gene diversity among populations ( $D_{ST} = H_T - H_S$ ) was 0.0349 (Table 3). Using these diversity indices it  
148 was possible to calculate the coefficient of gene differentiation ( $G_{ST} = D_{ST}/H_T = 0.1450$ ), which measures the  
149 proportion of total genetic diversity occurring among populations and is analogous to Wright's  $F_{ST}$  values.  
150 This means that only approximately 14.5% of the genetic variation observed in this study was due to  
151 differentiation among populations, while 85.5% is due to variation within populations,

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

156 AMOVA (Analysis of Molecular Variance) (Table 4) showed that 65% of the variability was  
157 within populations whilst 35% was among them. Analogous to Wright's  $F_{ST}$  is also the  $\Phi_{PT}$  value,  
158 estimated with AMOVA; it amounted to 0.345 ( $P >= 0.001$ ), based on permutation across the full data set.

159 To compare the degree of genetic differentiation among populations, the standard genetic  
160 distance of Nei (1972) was estimated. The value of Nei's genetic distance was  $D = 0.0881$ , while the

161 value of genetic identity was  $I = 0.9157$ , indicating low genetic differentiation between the two  
162 populations.

163

#### 164 **4. Discussion**

165 RAPD assay was used in order to analyze the genetic diversity and differentiation in two  
166 southern Portuguese populations of *Ruditapes decussatus*. The technique was found to be effective in  
167 revealing polymorphisms in this species, since all 10 random primers produced at least one polymorphic  
168 fragment. The major limitations of this technique are its lack of reproducibility (Ford-Lloyd and Painting  
169 1996; Weising et al. 1994; Guadagnuolo et al. 2001) and its sensitivity to small variations in PCR  
170 conditions. In order to overcome these limitations, DNA of high quality was used and three repetitions  
171 were performed for the same primers and individuals which generate the same results.

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

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

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

200 In conclusion, the two populations of *R. decussatus* studied presented high values of genetic  
201 variability within populations and low values of genetic differentiation between them, demonstrating a  
202 high degree of genetic homogeneity. The value of the gene flow ( $N_m$ ),  $G_{ST}$  and  $\Phi_{PT}$  quantification  
203 demonstrate that the exchange of genes between the two populations is high. A high degree of genetic  
204 homogeneity among populations has also been described for populations of other marine invertebrates,  
205 such as *Concholepas concholepas* (Gallardo and Carrasco 1996), *Ostrea chilensis* (Toro and Aguila  
206 1996) and *Venus antiqua* (Gallardo et al. 1998). The high values found for the genetic diversity within  
207 populations, associated with low values of genetic variation among populations, suggest panmixy as the  
208 dominant mode of reproduction (Armbruster 1997, 1998; Fernández-Tajes et al. 2007).

209 Mollusc species with extended larval planktonic phases are generally thought to disperse further  
210 and to show higher rates of gene flow, larger geographic ranges, lower levels of genetic differentiation  
211 among populations and high levels of genetic variation within populations (Scheltema and Williams  
212 1983; Waples 1987; Williams and Benzie 1993; Palumbi 1995). The life cycle of *R. decussatus* presents a  
213 larval planktonic stage, which lasts about three weeks, followed by benthic juvenile and adult stages.  
214 During the larval phase, larvae can travel many miles driven by currents before settling in favourable  
215 habitats.

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

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226



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234

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365 **Figure legends**

366 Fig. 1 Number of bands and mean heterozygosity in two *Ruditapes decussatus* populations.  
367

368 Table 1. Selected primers for the evaluation of genetic diversity in the two populations of *Ruditapes*  
369 *decussatus*  
370

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	380-1500
OPE-02	GGTGCGGGAA	36	15	300-880	10	520-1200
OPE-03	CCAGATGCAC	36	17	300-880	16	350-1100
OPE-06	AAGACCCCTC	36	22	250-1500	17	250-1100
OPE-10	CACCAGGTGA	36	17	250-1300	23	250-1500
OPE-11	GAGTCTCAGG	36	17	180-1200	18	180-1300
OPE-12	TTATCGCCCC	33	15	280-750	12	380-950
OPE-14	TGCGGCTGAG	33	20	320-1000	21	250-1000
OPE-15	ACGCACAACC	33	21	200-800	24	180-1000
OPE-16	GGTGACTGTG	36	12	320-850	21	200-1500

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373 Table 2. Genetic variation within two Portuguese populations of *Ruditapes decussatus*. (N - number of  
374 sampled individuals;  $n_a$  - Number of expected alleles;  $n_e$  - effective allele number;  $h$  - Nei's gene diversity;  
375  $H$  - mean Shannon's information index; %  $P$  - percentage polymorphic loci calculated with all alleles  
376 included;  $SE$  - standard error  
377

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	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	

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

382

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

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Table 4. Analysis of Molecular Variance (AMOVA) in the two Portuguese populations of *Ruditapes decussatus*

Source	df	SS	Variance (%)	Fixation index ( $\Phi_{PT}$ )
Among pops	1	463.320	35	0.345
Within pops	48	1569.520	65	
Total	49	2032.840	100	

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