GENETIC DIFFERENTIATION IN NATURAL AND CULTURED MUSSELS OF *Perna perna* (MOLLUSCA, MYTILIDAE) IN SANTA CATARINA

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ABSTRACT

The aim of this work was to analyse genetically natural beds and cultures of the most commercially important mollusc of Santa Catarina (South Brazil), the brown mussel *Perna perna*. Two sites were chosen for sampling from natural beds and for settling experimental lines with seeds obtained from each local farm. Experimental lines were maintained during four months for evaluating the growth of mussels from different cultures. DNA was extracted by Chelex-Proteinase-k method from adductor muscle and amplified by the Polymerase Chain Reaction (PCR) using PMS1 microsatellite primers. Denatured 6% polyacrylamide gel and silver staining were used for separation and visualisation of PCR products, respectively. The nuclear locus obtained showed no significant differences in allele frequencies between natural stocks. Cultured mussels in Penha did not differ genetically from natural beds, but Canto Grande culture was significantly different from the local stock. Mean sizes reached in experimental lines and also from individuals collected in natural stocks were found to be higher in Canto Grande than in Penha. Canto Grande samples showed departure from Hardy-Weinberg equilibrium at the studied locus, due to heterozygote deficiency. The genetic distinction of Canto Grande cultured mussels suggests that seeds from other localities may have been used occasionally by fishermen to set lines for culture.

Key words: nuclear DNA, microsatellite PMS1, Mytilidae, *Perna*, aquaculture.

DIFERENCIAÇÃO GENÉTICA DE ESTOQUES NATURAIS E CULTIVO DO MEXILHÃO *Perna perna* EM SANTA CATARINA.

RESUMO

O objetivo do presente trabalho foi de analisar geneticamente estoques naturais e de cultivo do molusco de maior importância econômica de Santa Catarina (Sul do Brasil), o mexilhão *Perna perna*. Dois locais foram escolhidos para a coleta de bancos naturais e para a instalação de cordas experimentais a partir de sementes obtidas do cultivo local. As cordas experimentais foram mantidas durante quatro meses para avaliação do crescimento dos mexilhões dos diferentes cultivos. O DNA foi extraído pelo método chelex-proteinase-k de músculo adutor e amplificado pela reação em cadeia da polimerase (PCR) utilizando oligonucleotídeos iniciadores (primers) para o microsatélite PMS-1. A separação dos produtos de PCR foi realizada num gel desnaturante de poliacrilamida 6%, os quais foram revelados com coloração de nitrato de prata. O loco nuclear obtido não mostrou diferenças significativas entre os estoques. De acordo com este loco, em Penha, os mexilhões cultivados não diferem geneticamente do estoque natural. No entanto, em Canto Grande estes diferem dos mexilhões do ambiente natural. Os tamanhos médios atingidos nas cordas experimentais e aqueles obtidos de indivíduos do ambiente natural foram maiores em Canto Grande que em Penha. As amostras de Canto Grande também mostraram provir de uma

população que se afasta do equilíbrio de Hardy-Weinberg, devido a uma deficiência de heterozigotos. A distinção genética dos mexilhões cultivados de Canto Grande sugere que sementes de outras localidades podem ter sido usadas ocasionalmente pelos miticultores para a implementação de cordas de cultivo.

Palavras Chave: DNA nuclear, microsatellite PMS1, Mytilidae, *Perna*, aquacultura

INTRODUCTION

The brown mussel *Perna perna* (Linnaeus, 1758) is the most commercially important mollusc in Santa Catarina, South Brazil, by direct extraction of adults from natural beds or by culturing them in protected areas of bays, through lines hanging from floats, using seeds extracted either from the surrounding natural stocks or those settled in the same lines. Santa Catarina is responsible for 97% of the production of molluscs in Brazil (Borghetti *et al*, 2003). The culture of *Perna perna* began in Santa Catarina in 1988, and since then its production has grown up steadily (Grumann et al, 1998; Costa *et al*, 1998). During 1998 and 1999 the production of cultured molluscs increased to 8,500 tons, involving about a thousand of fisherman families. In 2000 was observed the highest value of production (11,365 tons) since the initiation of the culture (Winckler, 2003), but afterwards the production start to decline steadily. As any other commercially exploited species, the brown mussel has been submitted to a high fishing effort, which could lead to the depletion of the natural stocks if no management measures are taken. The evaluation of the degree of conservation through genetic monitoring is needed for subsequent management. Therefore, the aim of the present work was to evaluate the degree of genetic diversity in natural and cultured stocks, and the population subdivision of two Santa Catarina stocks, which are submitted to slightly different environmental conditions (Resgalla Jr. *et al*, 1999). Allozyme markers has been used in previous studies for analysing the genetic structure of natural populations of *P. perna* (Silva, 1991; Grant *et al*, 1992; Neto, 2003) for the eastern and western Atlantic coasts, where moderate subdivision has been found. Holland (2001) described microsatellites loci for *P. perna*, from which he observed high polymorphism studying populations along the distribution range of the species. DNA-based markers in general are more variable than allozymes, therefore were chosen for the present purpose.

MATERIAL AND METHODS

Sampling

One hundred adult individuals were collected in August and October of 2001 from natural beds of the following sites of Santa Catarina state, Brazil: "Enseada de Armação do Itapocoroy" (26°48'S, 48°35'W) in Penha and "Zimbros, Canto Grande" (27°13'S, 48°30'W) in Bombinhas (Figure 1). In the same localities, were obtained 200 seeds, extracted from the lines of the established cultures, with sizes of 2-3 cm (maximum length of the shell) for settling four experimental lines at each locality. Tubes of PVC were used for adding the seeds within two kind of nets, the inner one of cotton and the outer of nylon. The lines were maintained during four months in the sea inside the established local cultures, from the 19th of December of 2001 until the 8th of May of 2002 in Penha and from the 20th of December of 2001 until the 24th of April of 2002 in Bombinhas. The sizes of all individuals were obtained after collecting them from the natural beds and at the end of the experiment, in the case of the experimental lines, by using a calliper (0.1 mm). STATISTICA for windows v 4.3, 1993, Statsoft, Inc., was used for comparing means between

localities and experimental lines by two-way ANOVA analysis.

Genetic Analysis

DNA was extracted from adductor muscle by using the Chelex 6%-Proteinase-K protocol described by Öhresser *et al*. (1997). Nuclear DNA fragments were amplified by the Polymerase Chain Reaction (PCR) using PMS1 primers, 5'-TCA TCT GTT GTT GTC TTT TTG-3' and 5'-GAC AAG AAG TTG ACT AGA ATA ATG-3', which were described by Holland (2001) for amplifying a TG-unit microsatellite locus specific to *P. perna*. The reaction mixture contained 1 μL of extracted DNA, 25 ng of each primer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 1 U of Taq DNA polymerase. Amplification by PCR was done programming the following steps in the thermocycler: initial denaturing temperature of 94°C for 2 min, 30 cycles of 94°C, 48°C, and 72°C for 30 s each, and a final extension of 72°C for 2 min. Products obtained by PCR were first checked in 1.5% agarose gel, using a 123 bp ladder, and afterwards were run in a 6% denaturing polyacrylamide vertical gel, using a molecular marker of 10 bp. A pre-run for warming the gel was done, before the electrophoresis of samples. Samples were mixed with formamide loading buffer and denatured during 5 min at 95°C before the electrophoresis. Electrophoresis conditions were adjusted by maintaining the power at 45 Watts during 1.5 h. Data analysis (allele frequencies, variability measures, pair-wise genetic identities and distances, and Chi-square test for the homogeneity in allele frequencies) was done using POPGENE program v. 3.1.1

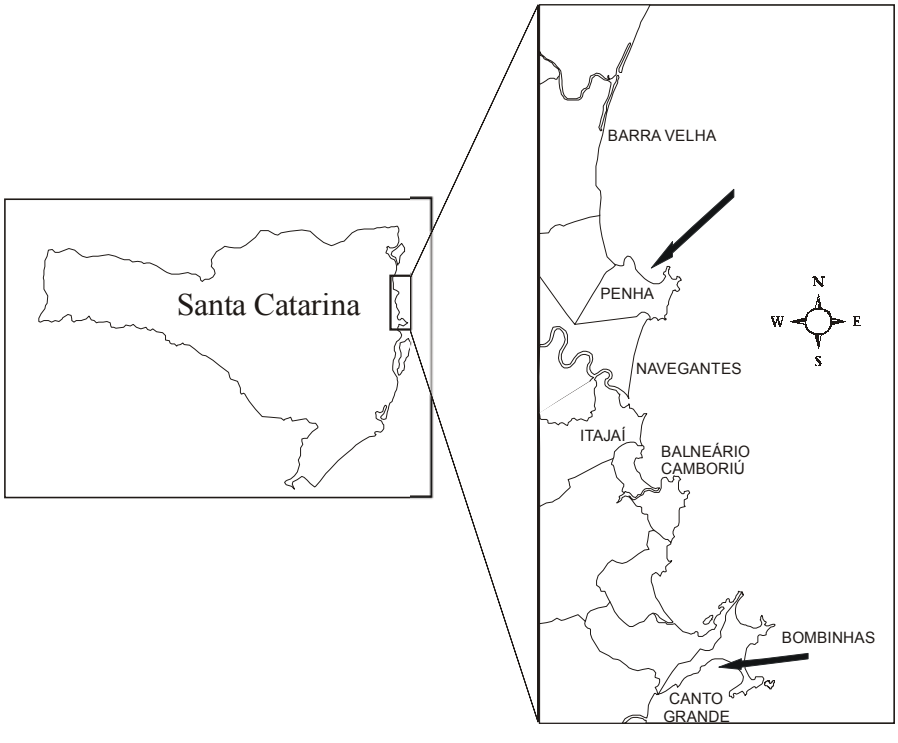

Figure 1 - *Perna perna*. Arrows indicate sampling sites and localities where experimental lines were settled.

(Yeh & Boyle, 1997). Also, FSTAT v. 1.2 program (Goudet, 1995) was used for obtaining theta, the estimator of FST, and its significance, as a measure of genetic subdivision between natural populations.

RESULTS AND DISCUSSION

Mean sizes

The mean sizes of adult individuals collected from the natural stocks and of those at the experimental lines are shown in Table 1. The differences observed between localities and among lines, are shown in Figure 2. The two-way ANOVA analysis showed that there is effect of localities and also of experimental lines for the growth of the brown mussel *P. perna* (Table 2), where the effect of lines was mainly due to the variation observed in Penha (Figure 2). At this site, lines were affected by the presence of epibionts, where those with higher number of epibionts showed mussels with reduced sizes and less individuals at the end of the experiment.

Allele frequencies and diversity at the nuclear locus

It was observed for all individuals the amplification of five regions, which were detected by agarose 1.5% gel. Only one of this region was coherent with a codominant locus and within the expected size of the microsatellite (minimum 224 bp with only one TG repetition), showing variants that differ in two base-pairs. Three alleles (260, 262 and 264 bp) were detected for this locus, which we expect correspond to the microsatellite variants with 19 to 21 TG repetitions (Figure 3). The

Table 1 - *Perna perna*. Mean sizes of adult individuals from the natural stocks, and at the experimental lines after four months in the sea.

Locality/type of sample	Total number of individuals	Mean size (mm)	Standard error (mm)
Penha - natural stock	50	64.43	1.22
Canto Grande - natural stock	50	70.12	1.17
Penha - experimental lines	115	41.03	0.83
Canto Grande- experimental lines	166	50.32	0.64

Table 2 - *Perna perna*. Two-way ANOVA for testing the effect of line and locality over the mean sizes reached after four months at the experimental lines.

Source	DF	SS	DF	MS	F	p
Locality	1	5751.00	273	70.27	81.84	<0.0001*
Line	2	191.34	273	70.27	2.72	0.0447*
Locality x Line	3	180.37	273	70.27	2.57	0.0549

* Significant at the level of α= 0.05.

microsatellite submitted to the NCBI gene bank by Holland on the 17th of February of 2000 (Access number AF236062) for PMS1 locus, obtained from an individual from Gulf of Mexico, showed 16 TG repetitions, with a fragment size of 254 bp when the sequence corresponding to the primers are included. Allele frequencies, variability measures, and χ^2 test of fitness to Hardy-Weinberg equilibrium (H-W) at this nuclear locus are presented in Table 3. Individuals from Canto Grande experimental lines differ from all other samples by showing a third allele (264 bp), which is also the most common allele, while the other samples showed the 262 bp allele as the most common (see Table 3). Canto Grande samples, both from experimental lines and natural stock, did not fit the H-W equilibrium, due to a strong deficiency of heterozygotes. But, those from Penha were in equilibrium. Heterozygosity and number of alleles, found at

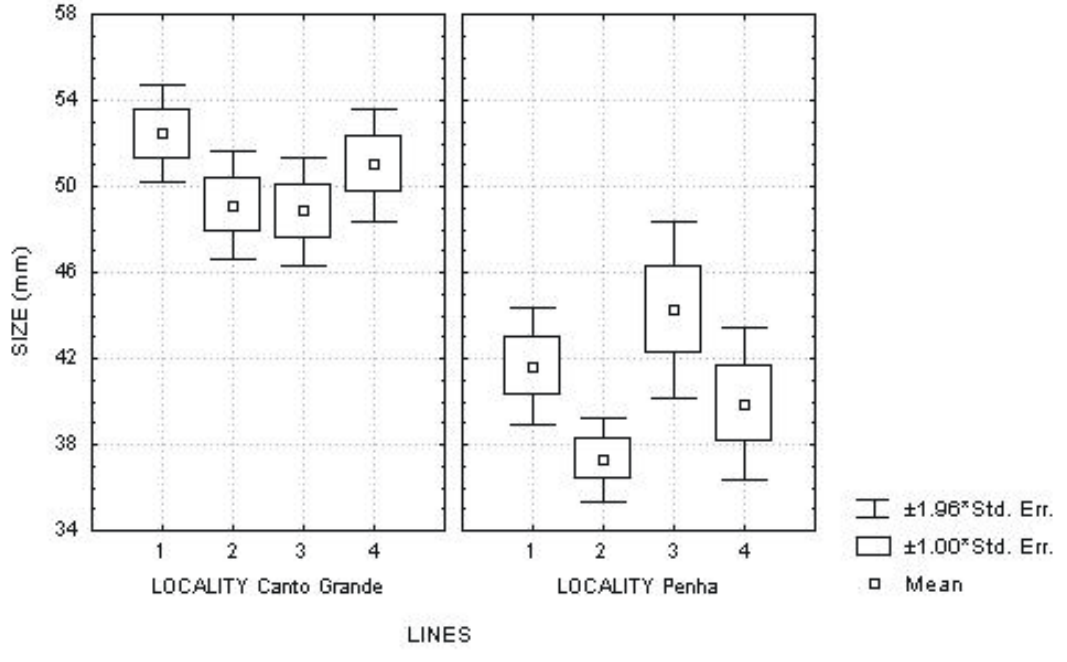

Figure 2 - *Perna perna*. Mean sizes of individuals after four months growing in the sea at each experimental line (1-4).

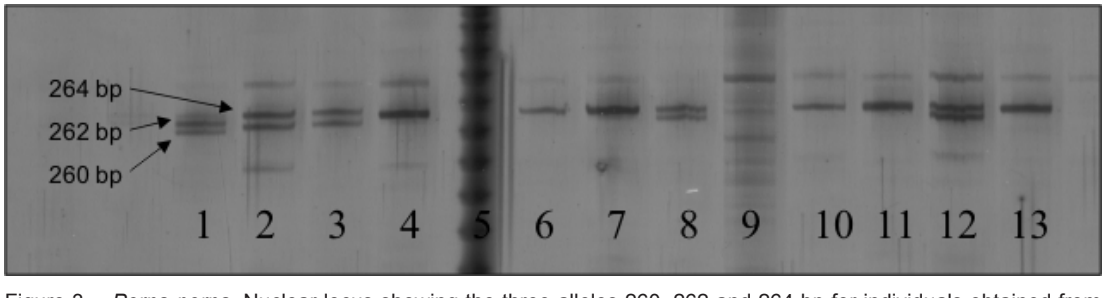

Figure 3 - *Perna perna*. Nuclear locus showing the three alleles 260, 262 and 264 bp for individuals obtained from the experimental lines of Canto Grande.

Table 3 - *Perna perna*. Allele frequencies, variability measures, and χ^2 test of fitness to Hardy-Weinberg equilibrium (H-W) at a nuclear locus for *Perna perna*.

Nuclear *locus*	Populations (N)			
	Penha		Canto Grande	
Allele in bp	Natural (48)	Cultured (46)	Natural (50)	Cultured (48)
260	0.333	0.261	0.420	0.145
262	0.667	0.739	0.580	0.355
264	0.000	0.000	0.000	0.500
Ho	0.333	0.348	0.200	0.375
He	0.444	0.386	0.487	0.603
F	0.250	0.098	0.589	0.378
χ^2 (H-W)	1.78	0.34	9.32	11.98
DF	1	1	1	3
p	0.181	0.558	0.002*	0.007*

(Ho) observed heterozygosity, direct count; (He) expected heterozygosity under H-W principle (Nei, 1973); (F) fixation index, a measure of heterozygote deficiency within populations.

the locus are very low when compared with data showed by Holland (2001), who found for a sample of 26 individuals of Rio Grande, 20 alleles, and an heterozygosity of almost 90%. These data seems to be extremely high, when compared with the literature, being at least rare. Curiously, we could not confirm the high variability found by this author for PMS1. For this locus, five regions were amplified for all individuals studied and detected by common agarose gel, using exactly the same conditions described by the author. This led us to search in the Genebank to verify the possible sizes of the microsatellite, showing that the microsatellite should be larger than 222 bp, which correspond to the size without any TG repetition. Sprecher et al (1996) showed that artefacts in polyacrylamide gels may lead to confusions in scoring alleles, and we wonder if this was not the case. Populations of *P. perna* in the surrounding coasts of Rio Grande are very scarce, and isolated, close to the end of the distribution of the species, and all registers south Rio da Prata are dubious (Neto, 2003), so it is very surprising to found such a high diversity in Rio Grande at PMS1 locus, where genetic drift should have an important effect in diminishing genetic diversity. We can conclude from the present work, that the primers for obtaining the PMS1 microsatellite described by Holland (2001) are not optimal for getting the PMS1 microsatellite, due to the amplification of other regions, that make very difficult to recognise and identify it properly.

Genetic differentiation between cultured and natural populations from north and central-north of Santa Catarina

Homogeneity χ^2 test for allele frequencies showed that the only population that differed from all other was Canto Grande cultured group (Table 4). Genetic identity confirm this view by showing Canto Grande experimental lines separated from the other samples by more than 0.48 of genetic distance (Table 5 and Figure 4). The high genetic identity between both kind of populations (natural and cultured) from Penha, demonstrated that cultured individuals in this area are very representative of their natural stock. No significant differences were found between north and central-north natural populations of Santa Catarina (Table 4), which was also confirmed by non-significant theta (θ = -0.014; p= 0.402). This confirm the view of previous authors, of the lack of genetic structure in *P. perna*, in the absence of physical or oceanographic barriers, over moderate distances (Silva, 1991; Neto, 2003).

The low increments in size observed for Penha mussels might be the consequence of the disturbance of epibionts or perhaps, differences might be a physiological response to different environmental characteristics, as was previously suggested by Resgalla Jr. *et al.*

Table 4 - *Perna perna*. Test of the (2 for the homogeneity in allele frequencies between samples.

Samples	χ^2	DF	p
Canto Grande: culture x natural	34.10	2	< 0.001*
Canto Grande, natural x Penha, natural	0.78	1	0.376
Penha: culture x natural	0.50	1	0.442

* Significant at the level of α= 0.05.

Table 5 - *Perna perna*. Nei's (1978) genetic identity (above the diagonal) and distance (below the diagonal) between pair of samples.

Samples	Penha		Canto Grande	
	natural stock	experimental lines	natural stock	experimental lines
Penha, natural stock	****	0.999	0.996	0.614
Penha, experimental lines	0.000	****	0.967	0.614
Canto Grande, natural stock	0.004	0.034	****	0.599
Canto Grande, experimental lines	0.488	0.487	0.513	****

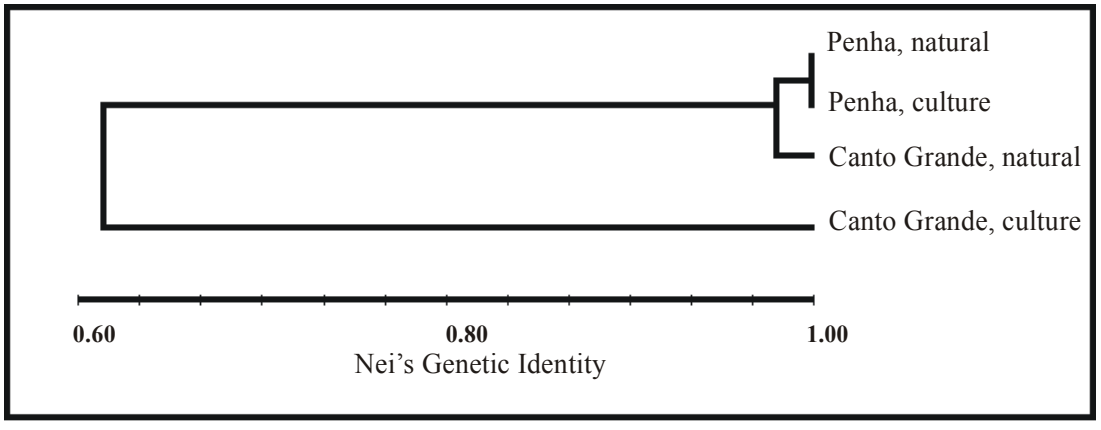

Figure 4 - *Perna perna*. The UPGMA cluster, based on Nei's (1978) genetic identity, showing the similarity between experimental lines and natural stocks of the different localities.

(1999), after studying growth rates and other physiological parameters in *Perna perna*. Nonetheless, the genetic differences found between mussels from the experimental lines of Canto Grande and Penha, does not allow us to rule out the possibility of a genetic basis on the differences found in growth rates between mussels coming from different sites. The genetic differences detected between them may also be temporal variation in allele frequencies, if seeds from other populations are occasionally incorporated in Canto Grande cultures by fishermen. This could also explain the deviation from Hardy-Weinberg equilibrium in Canto Grande. Larger number of loci, monitoring during longer periods of time, and the use of more variable neutral loci will be necessary to confirm any genetic basis for differential growth rates among cultures from the north and central-north of Santa Catarina.

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