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Temporal genetic differentiation in cultured and natural beds of the brown mussel *Perna perna* (Mytilidae)

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ABSTRACT. *Perna perna* is the most important cultivated mussel of Santa Catarina, Brazil, sustaining an important economic input for many local families. Natural stocks of *P. perna* are depleted by the extraction of adults and seeds for consumption and culture. The aim of the present study was to use the microsatellite locus *pms-2* to study the variation of the genetic composition and diversity between natural and cultured stocks in samples of 2001 and 2005 from Penha, Santa Catarina. DNA was extracted from adductor muscle by Chelex/proteinase-K and phenol/chloroform protocols. Amplification by polymerase chain reaction was performed using specific primers for analyzing the *pms-2* locus. Polymerase chain reaction products were submitted to vertical denatured 6% polyacrylamide gel electrophoresis and horizontal 2% agarose gel electrophoresis, and visualized by silver staining and ethidium bromide, respectively. Allele diversity and heterozygote deficiency were higher for samples of 2005 than for those of 2001. No significant genetic differentiation was found between natural and cultured stocks of 2001 by the χ^2 test, but G^2 (likelihood ratio) detected slight differences (I = 0.949; χ^2, P = 0.147; G^2, P = 0.046), while cultured and natural stocks of 2005

were very different (I = 0.798, P = 0.006). Between the years of 2001 and 2005, a large change in genetic composition was observed (I = 0.582; P < 0.001). Although nothing is known about natural changes in the genetic composition of this species with time, the results suggest a strong impact of human activities on natural stocks of *P. perna*, which is expected to be related to heavy extraction and farming.

Key words: Mollusca, Mytilidae, *Perna perna*, Population genetics, Microsatellites, Brazil

INTRODUCTION

Perna perna (Linnaeus, 1758) shows a wide geographical distribution, all along the coast of Africa and in America, it is found along the Brazilian coast and in the Gulf of Mexico, where it was recently introduced (Holland, 2001). It lives on exposed rocky shores from mid to low intertidal levels, as well as below the tidal level. This species is the most important cultured mussel of Santa Catarina, Brazil, sustaining an important economic input for many local families. This species is exploited by the extraction of adults either for direct consumption or for sale in the local community, and by the production and sale of seeds extracted from natural stocks, which are grown in lines distributed along protected areas of bays. After the establishment in Santa Catarina of the culture of *P. perna*, a maximum production was first reached in the year of 2000 with 11,365 tons. Afterwards, a steady decline in production was observed, reaching a minimum of 8,132 tons in 2003 (Oliveira Neto, 2006). In the last few years, strong production has been reestablished, probably due to the higher number of people involved in the activity, reaching in 2005 a production of 12,234 tons (Oliveira Neto, 2006). These numbers reflect the increase of cultured stocks, but do not necessarily reflect better conditions of natural stocks. Since this species has been heavily exploited, a decline in natural stocks is expected. We should then expect, if there is any effect from human activities related to the culture, to at least find differences in genetic composition between some years. Samples collected in 2001 from natural stocks of two localities (Bombinhas and Penha), situated in the central-north region of Santa Catarina and less than 50 km apart, showed homogeneity in allele frequencies at the microsatellite *pms-1* (Noel et al., 2004). This result was expected due to the lack of barriers to gene flow between the two locations. Nonetheless, when comparing cultured with natural stocks of each area, they found that the cultured stocks of the southern location showed great genetic differences from the other stocks, suggesting the incorporation of seeds from other locations in the southern culture. This possible introduction of seeds does not seem to have occurred until 2001 for the locality of Penha, which showed almost identical genetic composition between natural and cultured stocks. Although Holland (2001) described the *pms-1* locus as highly variable, Noel et al. (2004) did not find the same level of variability at this locus. Therefore, our aim was to test the *pms-2* locus to find out if a more sensitive marker would be able to detect differentiation between natural and cultured stocks collected from Penha in 2001 and to determine any differences between samples collected in 2001 and 2005 from the same locality.

MATERIAL AND METHODS

One hundred and nine individuals of *Perna perna* were analyzed from natural stocks of Praia Vermelha and from cultured stocks situated in the Bay of Armação do Itapocoroy, both situated at the locality of Penha (26°48'S, 48°35'W), which were sampled in August and October 2001 and in April 2005 (Figure 1).

Figure 1. Sampling site of *Perna perna*, indicated by an arrow.

DNA from samples of 2001 were extracted by the Chelex/proteinase-K protocol (Daguin and Borsa, 1999) from adductor muscle, while DNA from samples of 2005 were obtained from the same tissue but using the phenol/chloroform method (Caetano-Anoles and Gresshoff, 1997), showing the last method to be more efficient for obtaining DNA of good quality. For Chelex/proteinase-K DNA extraction, muscle tissue was macerated in 300 µL of lysis buffer (0.1 mM Tris-HCl, 0.01 mM EDTA, pH 8.0) containing 6% Chelex 100 (Sigma) resin and 60 µL proteinase-K (10 mg/mL), and incubated overnight (~15 h) at 56°C. The next day, samples were submitted to 95°C for 20 min. Then, supernatants were transferred to new tubes and subsequently used for amplification. For DNA extraction by phenol/chloroform, samples were macerated in 700 µL of lysis buffer (50 mM Tris-HCl, 25 mM EDTA, pH 8.0, 1% SDS) containing 50 µL proteinase-K (10 mg/mL) and incubated overnight at 37°C. The next day, 700 µL phenol/chloroform/isoamyl alcohol (25:24:1) was added, and the mixture was homogenized in a stirring

wheel for 10 min. After centrifugation at a maximum speed (12,000 *g*) for 5 min, the aqueous phase was transferred to new tubes. DNA was submitted to two further washes with an equal volume of chloroform/isoamyl alcohol (24:1) following the same steps as the previous wash with phenol. Afterwards, DNA was precipitated from the last aqueous phase with two volumes of cold absolute ethanol. To enhance precipitation, samples were incubated overnight at -20°C and on the next day, centrifuged at maximum speed for 18 min. Afterwards, the DNA pellet was washed three times with 70% ethanol, dried and resuspended in 120 µL TE (1 mM Tris-HCl, 0.5 mM EDTA) at 56°C for 30 min. Extracted DNA was submitted to RNAase using 2.5 µL RNAase (20 mg/mL) and incubated at 37°C for 30 min. The microsatellite locus *pms-2* was amplified by the polymerase chain reaction (PCR) using primers described by Holland (2001) for *Perna perna* (F: 5'-CGTCTCCATCTTTAATTACTA-3' and R: 5'-GCGCACTGTCAATG TT-3'). PCR reaction was performed using 1-5 µL extracted DNA (~10-50 ng), 2.5 µL PCR buffer 10X, 1.25 µL 50 mM $MgCl_2$, 0.24 µL mixed 100 mM dNTPs, 0.1 µL 100 µM of each primer and 0.2 µL Taq DNA polymerase (5 U/µL) (Invitrogen), which was completed with ultra-pure water (Sigma) to a final volume of 25 µL. The Thermocycler was programmed for the following steps: 1 cycle at 95°C for 3 min; 35 cycles at 94°, 48° and 72°C, each of them for 45 s, and a final extension at 72°C for 1 min. Platinum Taq DNA polymerase (Invitrogen) was used for improving the specificity of the reaction. PCR products were submitted to vertical denatured (6 M urea) 6% polyacrylamide (19:1) gel electrophoresis for separation of fragments, which were stained with silver nitrate following Hoelzel (1998) with modifications. A pre-run of about 1.5 h without samples was carried out at 70 watts in order for the gel to reach a temperature of 65°C. Afterwards, samples were denatured in formamide loading buffer at 95°C for 5 min, and immediately placed on ice and loaded into the wells of the gel. The electrophoresis run with the samples lasted 3 h at 45-50°C and 45 watts. The alleles were recognized by their size when compared to a 10-bp molecular size marker (Invitrogen), which was also denatured before loading into the wells of the gel. In order to save time and reagents, alleles were checked at the same time in a horizontal 2% long agarose gel with an electrophoresis run of 2.5 h. As most alleles were easily recognized by 2% agarose electrophoresis, we decided to score them by this method. Those alleles that were not easily distinguished in agarose gels were pooled for genetic analysis. A 100-bp molecular marker and alleles already recognized by vertical polyacrylamide gel electrophoresis were used for allele identification by agarose gel electrophoresis. Analyses of data were conducted using POPGENE (Yeh and Boyle, 1997).

RESULTS

Alleles showed sizes between 100 to 292 bp, and their frequencies are shown in Table 1. The locus was highly variable, where most alleles were easily identified by agarose gel electrophoresis (Figure 2). Many nonspecific bands were observed, which tend to disappear when using Platinum Taq DNA polymerase, and were more frequent in the range of 200-400 bp. The most frequent alleles of 2001 samples were different from those of 2005, but natural and cultured stocks from the same year showed the same most common alleles. Variability measures and G^2 (likelihood ratio) for goodness of fit to Hardy-Weinberg (H-W) equilibrium are shown in Table 2. All samples were highly variable, but even so they showed some heterozygote deficiency. When determining goodness of fit to H-W equilibrium

with χ^2 test, we found that most samples deviated from the equilibrium. The likelihood ratio test showed that only the cultured stock of 2005 deviated significantly from H-W equilibrium (Table 2). This stock showed also the highest values of heterozygote deficiency (F_{IS} = 0.543). In cases with many alleles at low frequencies, the use of the likelihood ratio test is recommended. This situation can be observed when comparing number of observed alleles with effective number of alleles. Effective number of alleles will decrease with the increase of observed alleles at low frequencies.

Table 1. Alleles and pooled ones and their frequencies at *pms-2* locus for the mussel *Perna perna* in Penha, Santa Catarina, Brazil.

Alleles		Stocks			
Code(*)	Size (bp)	2001		2005	
		Natural (2N = 40)	Cultured (2N = 58)	Natural (2N = 60)	Cultured (2N = 60)
A (2)	100-104	0.1000	0.0000	0.0000	0.0333
B (2)	108-112	0.0750	0.0000	0.0833	0.0333
C (2)	118-122	0.2250	0.2241	0.0333	0.1167
D	130	0.0500	0.0345	0.1000	0.1333
E (2)	138-140	0.0250	0.0000	0.1500	0.0167
F (2)	144-146	0.0000	0.0000	0.1833	0.1667
G	150	0.1250	0.1380	0.0333	0.0000
H	156	0.0000	0.0172	0.0667	0.0167
I	160	0.0000	0.0000	0.0000	0.0833
J	166	0.0000	0.0000	0.0500	0.0000
K	170	0.1000	0.2070	0.1167	0.1500
L	180	0.1250	0.1724	0.0500	0.1667
M	190	0.0250	0.0690	0.0833	0.0667
N	200	0.0000	0.0172	0.0333	0.0000
O	214	0.1250	0.1034	0.0167	0.0167
P	230	0.0250	0.0000	0.0000	0.0000
Q	292	0.0000	0.0172	0.0000	0.0000

*Number of pooled alleles.

Genetic differentiation was found among all stocks (G^2 = 143.84, P = << 0.0001). No significant differentiation was found between natural and cultured stocks of 2001 if χ^2 test was considered (P = 0.147). Nonetheless, use of the likelihood ratio test (G^2) indicated slight genetic differentiation (Table 3). Other comparisons showed high genetic differentiation between the stocks of 2001 and 2005, with significant differences between both natural stocks and the two cultured stocks (Figure 3, Table 3). Pair-wise genetic identities (Nei, 1978) are also presented in Table 3, showing that the genetically most similar stocks were those of 2001 and the most divergent were the natural stocks of different years.

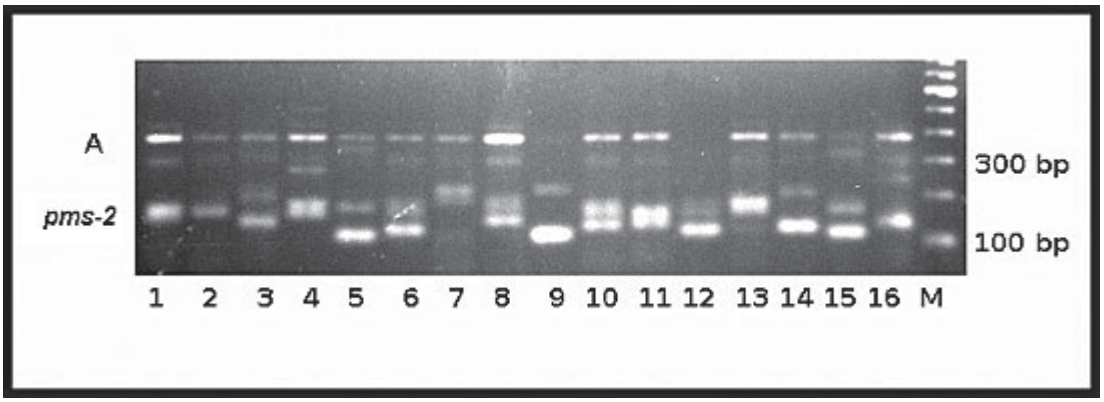

Figure 2. Alleles of *pms-2* locus and non-specific fragments detected by 2% agarose gel electrophoresis and visualized with UV light. Alleles of *pms-2* are localized in the lower region of the gel (*pms-2*), while most non-specific fragments are localized in the range of 200-400 bp (A). *Lanes 1* to *16*: samples from Penha culture of 2001; M, 100-bp molecular marker.

Table 2. Variability measures and G^2 test (likelihood ratio) for the goodness of fit to Hardy-Weinberg equilibrium (H-W) in natural and cultured stocks of *Perna perna* in 2001 and 2005.

Variability measures and G^2 test of H-W	Stocks			
	2001		2005	
	Natural	Cultured	Natural	Cultured
Number of alleles	11	10	13	12
Effective number of alleles (Kimura and Crow, 1964)	7.8	6.3	9.4	8.1
[1]Observed heterozygosity	0.550	0.690	0.733	0.400
[2]Expected heterozygosity	0.872	0.841	0.893	0.876
G^2 (likelihood ratio)	58.2	37.9	75.0	92.0
Degrees of freedom	55	45	78	66
P	0.359	0.765	0.575	0.019*

[1]Direct count; [2]expected by H-W (Nei, 1973); *Significant at P < 0.01.

Table 3. Comparisons between pair of stocks. Nei's (1978) genetic identity (above the diagonal) and probability (P) of the G^2 (likelihood ratio) test for homogeneity in allele frequencies (below the diagonal) between stocks.

Stocks	Natural 2001	Cultured 2001	Natural 2005	Cultured 2005
Natural 2001	-	0.949	0.454	0.676
Cultured 2001	0.046*	-	0.468	0.730
Natural 2005	<0.001***	-	-	0.798
Cultured 2005	-	<0.001***	<0.001**	-

Significant at: *P < 0.05, **P < 0.001, ***P < 0.00001.

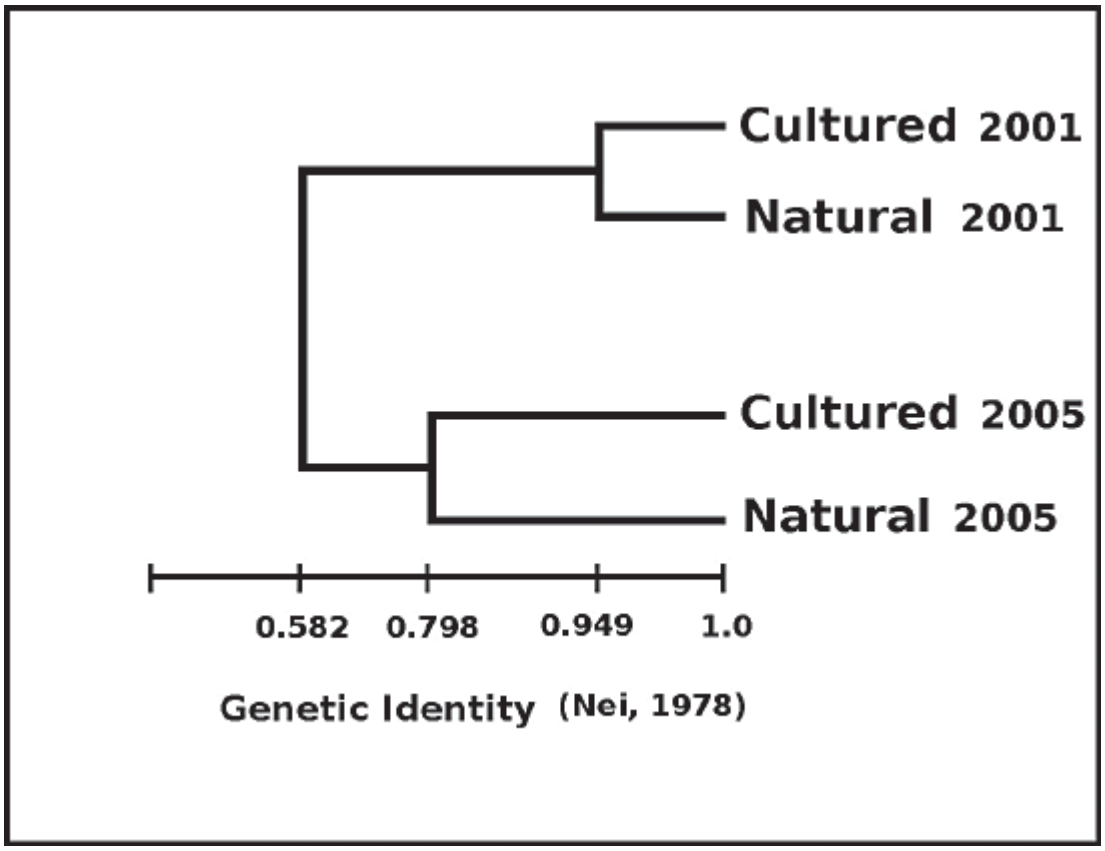

Figure 3. Dendrogram constructed using UPGMA (unweighted pair-group methods with arithmetic averaging) algorithm and Nei's (1978) genetic identity, showing the global similarity between natural and cultured stocks of *Perna perna* for 2001 and 2005.

DISCUSSION

High allele diversity was found for the *pms-2* locus when compared to values found for *pms-1* by Noel et al. (2004), showing that *pms-2* is a better molecular marker for population genetic analysis than *pms-1*. Observed (Ho = 0.400-0.733) and expected values (He = 0.841-0.893) of heterozygosity found in the present study are lower than those presented by Holland (2001). He found very high values of diversity for Brazilian populations (Ho = 0.930-0.957), for example, 21 alleles in 20 individuals sampled in a locality of the southernmost State of Brazil (RS), what is at least uncommon for a population situated close to the end of the distribution area of the species.

Microsatellites almost always reveal higher values of diversity than allozymes. Nonetheless, *Perna perna* has shown high values of allozyme diversity, such as those observed for the Brazilian (SP to RS, RJ) and African coasts (Ho = 0.320-0.582) (Silva, 1991; Moura-Neto, 2003).

Deviation from H-W equilibrium in the cultured stocks of 2005 was explained by a high deficit of heterozygotes. Heterozygote deficiency has been found to be very frequent in populations of marine mollusks, and has been explained by null alleles, inbreeding, selection, Wahlund effect, and aneuploidy (Fujio et al., 1983; Beaumont and Zouros, 1991; Corte-Real, 1992; Corte-Real et al., 1996; Weber and Hawkins, 2006). *Mytlilus galloprovincialis* from northwest Africa showed slight heterozygote deficiency at the nuclear locus *mac-1*, which was not caused by null alleles (Daguin and Borsa, 1999). In a northern population of New Zealand, strong heterozygote deficiency was found in *Perna canaliculus*, which was observed in only two allozyme loci (Apte and Gardner, 2001). Pompa et al. (1990) also found for Venezuelan populations of *Perna perna* heterozygote deficiency in a couple of allozyme loci, while an excess of heterozygosity was found in other loci. No heterozygote deficiency in allozyme loci was found in *Perna perna* populations of southern Africa (Grant et al., 1992). Holland (2001) did not find heterozygote deficiency at *pms-1* and *pms-2* loci, neither for natural stocks nor for introduced ones, in the Gulf of México. Nonetheless, Noel et al. (2004) found strong heterozygote deficiency for 2001 natural and cultured stocks of Canto Grande, Bombinhas (south of Santa Catarina State) at *pms-1* locus, but not for the northern stocks studied.

It is unknown to what extent the excess of homozygotes found at Penha (Santa Catarina, Brazil), in the cultured stock of 2005, is the result of the repeated introduction of seeds from other localities for the building up of lines (Wahlund effect). Should not these introductions have also affected the natural stocks of the surroundings due to the release of seeds carrying genetic differences? Sekino et al. (2005) found, in a preliminary study with microsatellites, that the release of seeds from cultures into natural stocks produced non-random mating and no heterozygote deficiency at multiple loci was found as should be expected.

It is less probable that the heterozygote deficiency detected in the culture of 2005 was the result of inbreeding, because a great proportion of seeds (normally all) came from natural stocks of the surrounding rocky shores, and mussel culture consists of raising seeds to commercial sizes on lines localized in protected areas. Less frequently, lines are prepared using seeds established in the same units of culture. Probably, in these cases, seeds are descendants of adults of the same lines and neighboring ones. Therefore, the following generations may have some percentage of inbred descendants by the union of gametes of first-degree relatives, but it seems to be rarely the case, since seeds from natural stocks are mainly used.

The high temporal variation observed between stocks of 2001 and 2005 is evidence in favor of the Wahlund effect hypothesis by the introduction of seeds from other areas.

Noel et al. (2004), studying *pms-1* locus, found that both stocks, natural and cultured, were almost identical in 2001, showing that until that time all seeds came from natural stocks. This point was corroborated by our data on *pms-2*, which showed only slight differences between these stocks, when using the likelihood ratio test, and no significant differences when using the χ^2 test. We also detected a huge difference between both kind of stocks four years later (in 2005) (see Figure 3), and a very high temporal differentiation between 2001 and 2005 for both kinds of stocks (I = 0.582). Manzoni (2005) reported that at the end of 2001 and beginning of 2002 fishermen started to incorporate seeds from other localities into the local culture of Penha. In addition, in 2003 it was observed a huge mortality of mussels in the culture. The cause of that depletion is not clear until now. The impact of farming over natural stocks has been already observed by the distribution of hybrid forms due to translocation of seeds in *Mytilus* spp (Smietanka et al., 2004) and by non-random mating as detected by microsatellites in the Ezo

abalone (Sekino et al., 2005). There is no doubt that human intervention makes an important contribution to the temporal genetic differentiation observed in Penha natural and cultured stocks of *P. perna*. Further studies will be necessary to answer the question of how much of the observed temporal variation between 2001 and 2005 is due to anthropogenic effects and how much is due to natural processes.

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