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CELLULAR AND MOLECULAR BIOLOGY

Genetic divergence accessed with microsatellite markers reflects the time of *Crassostrea gigas* genetic breeding in Brazil

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Abstract: The Pacific Oyster was introduced on Santa Catarina Island in 1987, experiencing processes of selection and genetic breeding since then. Such procedures may have led to the establishment of specific strains, given the saltier and warmer conditions of the Atlantic Ocean. This study employed microsatellite markers to compare allelic patterns of oysters cultivated in Santa Catarina, the USA, and Asia. Specific allelic patterns were revealed in the Santa Catarina samples, reflecting the time of selection/breeding of the oyster in this region. This result supports the effectiveness of the selection/breeding procedures and the demand for protection of this commercially important genetic resource.

Key words: Genetic structure, reproductive isolation, stepwise mutation model, evolutive biology.

INTRODUCTION

The Pacific Oyster [*Crassostrea gigas* (Thunberg, 1793)], natively distributed along the Asian coast, is one of the most important bivalves produced throughout the world (FAO 2016). Introduced in several countries, including New Zealand, Australia, South Africa, France, Canada, the USA, Chile, and Brazil, the large demand and exploitation of this marine mollusk accelerated the development of genetic management practices and breeding programs.

In Brazil, the state of Santa Catarina responds to about 97.2% of commercial mollusk production and is the country's main producer of *C. gigas* (Souza et al. 2022). Around 83% of the total *C. gigas* produced in the state originate from farming structures established in the South and North Bays of Santa Catarina Island (MAPA 2019). This region of the state was the pioneer in *C. gigas* farming, starting in 1987 and, since then, oyster farmers and researchers have been dedicated to selecting and improving this crop. This practice delivered management techniques and selection of matrices that allow the whole cultivation process, from the introduction of the "seeds" to the harvest, to be completed in 6 to 10 months in a saltier and warmer marine environment.

In this study, microsatellite markers were employed to characterize the allelic patterns of *C. gigas* cultivated on Santa Catarina Island, testing the hypothesis that the oysters produced in this region have specific allelic patterns due to selection and reproductive isolation concerning other regions of oyster production in the world.

MATERIALS AND METHODS

Samples of *Crassostrea gigas* (Figure 1a) were collected from marine farms in the South (27°49'S 48°33'W) and North Bays (27°30'S 38°41'W) of Santa Catarina Island (Figure 1b). All

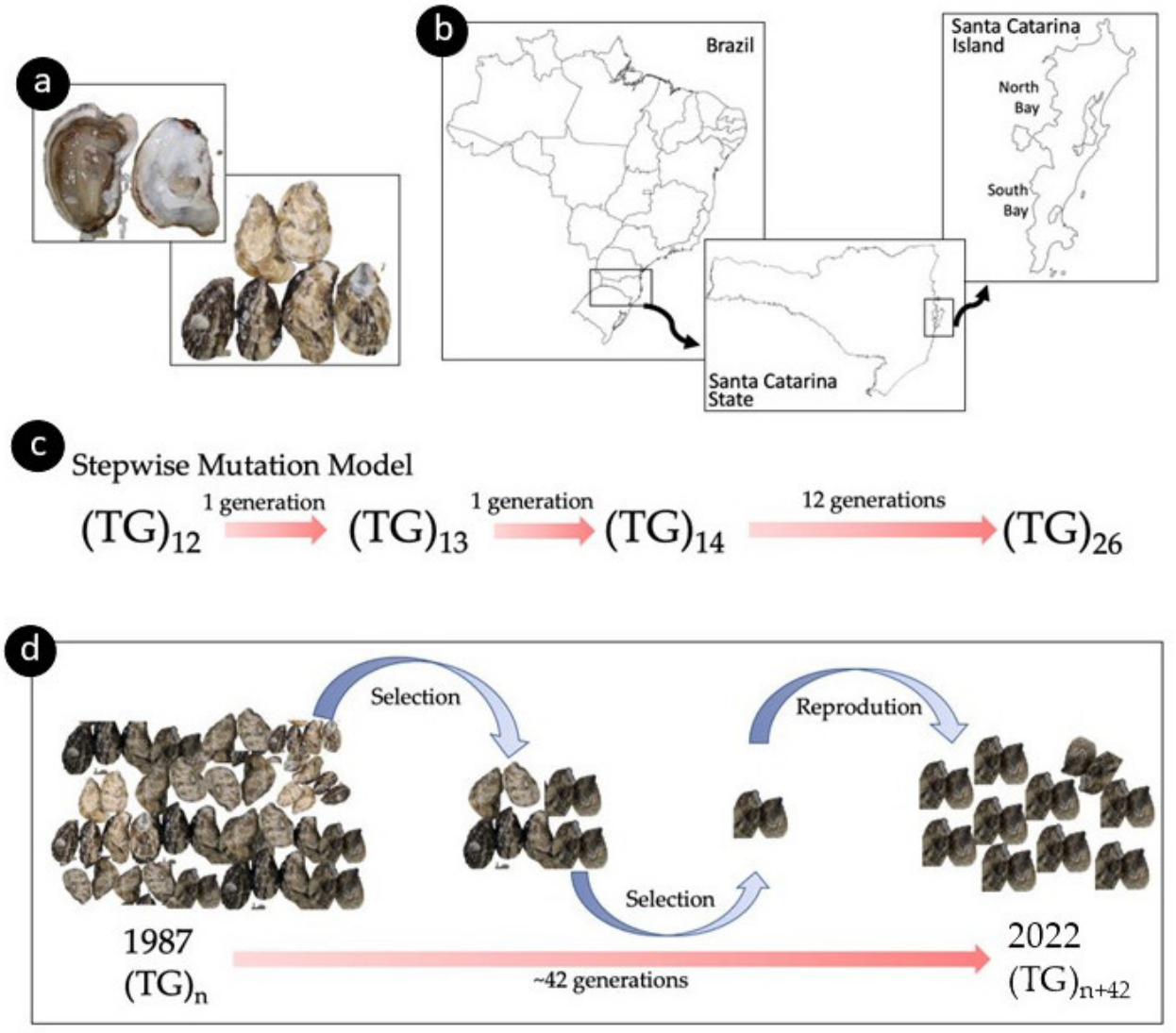

Figure 1. Correlation among oysters genetic breeding and molecular evolution. (a) *C. gigas* genotypes collected in Santa Catarina Island. (b) Map of study sites in Brazil, detailing the location of Santa Catarina State and the Santa Catarina Island, the North Bay, and the South Bay. (c) Diagram of the stepwise mutation model for a microsatellite marker with TG motif of repetition. In this example, at each generation, the allele increases one motif. (d) Representation of the selection process of C. gigas promoted since 1987, selecting oysters adapted to the saltier and warmer conditions of the Atlantic Ocean in Santa Catarina Island.

oysters cultivated in these regions are supplied by the Laboratory of Marine Mollusks of the Federal University of Santa Catarina (LMM/UFSC), which develops the selection process, genetic breeding, and production of *C. gigas* seeds. Sampling these two regions covers the genetic characteristics of the oysters cultivated in Santa Catarina since all the seeds are distributed by the same laboratory, from the same breeding program.

Approximately 100 mg of abductor muscle or gills (30 samples/site) were isolated, individually conditioned in 2.0 mL microtubes containing the lysis buffer (Li et al. 2006) and kept at 21±2 °C for 24 hours. The lysis product was extracted with phenol:chloroform and the total DNA precipitated with ice-cold isopropanol

(-20 °C) and washed sequentially with 70% and 100% ice-cold ethanol. The concentration and purity of the DNA were evaluated using a NanoDrop® spectrophotometer.

Nine microsatellite markers developed for *C. gigas* [Crgi39, Crgi45, Crgi4 (Sekino et al. 2003), otgfa00007B07, otgfa00129E11 (Qi et al. 2009), ucdCg-170, ucdCg-156, ucdCg-199, and ucdCg-152 (Li et al. 2003)] were PCR amplified (50 ng of DNA, 1× of PCR buffer, 0.5 µM of $MgCl_2$, 1 U of Taq DNA-Polymerase, 0.05 µM of each dNTP, 0.125 M of each forward and reverse primers, and 0.125 µM of fluorescently labeled M-13 primer $^{6\text{-HEX}}$5'-TGTAAAACGACGGCCAGT-3') using a Veriti™ thermocycler (Thermo-Fisher Scientific). All forward primers carry the M-13 sequence (5'-TGTAAAACGACGGCCAGT-3') in its 3' portion (Schuelke 2000). The amplification cycle consisted of 5 minutes at 94 ºC, 30 cycles of 30 seconds at 94 °C, 30 seconds at 50 °C and 1 minute at 72 °C, and 10 cycles of 1 minute at 94 °C, 1 minute at 53 °C and 1 minute at 72 °C, ending with one step of 10 minutes at 72°C. PCR products were separated in an ABI 3500xL® Genetic Analyzer and the alleles call was performed using the GeneMapper™ software (Applied Biosystems).

The genetic diversity of the samples from Santa Catarina was estimated based on the total number of alleles (A), the effective number of alleles (A_e), the observed (H_O) and expected (H_E) heterozygosities, and the within-population fixation index [$F_{IS} = (H_E - H_O)/H_E$]. The genetic structure between North and South Bays samples was evaluated using the AMOVA approach. All estimations were performed using GenAlEx 6.5 (Peakall & Smouse 2012).

Considering the hypothesis that the selection of oysters more adapted to the Atlantic Ocean in Santa Catarina Island retained advantageous alleles, which differ from the alleles preserved in oysters growing in the Pacific Ocean conditions, the allelic pattern of Santa Catarina oysters was compared with allelic data obtained in the literature for *C. gigas* cultivated in the USA (Li et al. 2003), Japan (Sekino et al. 2003), and China (Qi et al. 2009 and Liu et al. 2017).

RESULTS AND DISCUSSION

The levels of genetic diversity in North and South Bays were highly similar concerning the mean number of alleles (A = 1.8 and A = 1.9, respectively) and the effective number of alleles (A_e = 1.6 and A_e = 1.4, respectively). On the other hand, the South Bay revealed a lower estimation of H_O and H_E, and a higher fixation index (H_O = 0.08, H_E = 0.25, F_{IS} = 0.42) than the North Bay (H_O = 0.22, H_E = 0.36, F_{IS} = 0.33). These estimations are lower than the mean values reported for wild populations and breeding stocks from China, Japan, and the USA (A = 7.4, H_O = 0.56, H_E = 0.66). Nevertheless, ongoing studies have shown somewhat higher levels of diversity in the most recent *C. gigas* genetic stock of the LMM/UFSC (A = 8, A_e = 3.6, H_O = 0.53; unpublished data), an important point for the stability and progress of the breeding program.

According to the AMOVA, the main component of the total variation is found among individuals (74%), followed by the variation between North and South Bays (16%) and the within-individual variation (11%).

Studies of genetic diversity based on microsatellite markers have reported lower levels of genetic diversity in domesticated populations of *C. gigas*, as for mass-selected orange-shell lines (A = 3.98, H_O = 0.60, and H_E = 0.49; Han et al. 2019) and distinctive shell colors strains (A = 5.93, Ae = 4.7, H_O = 0.62, and H_E = 0.67; Zhang et al. 2023) in comparison to wild stocks. Both studies revealed higher values in comparison to the evaluated samples from Santa Catarina Island. Such higher estimations

are likely due to the larger and more diverse base populations used to establish the full-sib families employed in the genetic improvement programs (Han et al. 2019, Zhang et al. 2023).

The similarity in levels of genetic diversity parameters and the high estimations of the fixation index in the populations from Santa Catarina Island are the result of the genetic improvement program that selects the genotypes more adapted to the environmental conditions of the cultivation areas. In comparison to the oceanic conditions in Asia and the USA, the Atlantic Ocean in Santa Catarina Island presents saltier and warmer conditions. Thus, the most adapted alleles for these conditions tend to be selected and fixed in the breeding population.

For microsatellite markers, the different alleles in a population/lineage are determined by their size in base pairs. Considering the stepwise mutation model (SMM; Kimura & Ohta 1978) for the origin of new alleles, the size of the allele increases or decreases one motif per generation (Figure 1c). Thus, the larger the difference in size between two alleles, the longer the time that separates two populations/lineages. Ten alleles from five loci (ucdCg-170, ucdCg-156, ucdCg-199, Crgi39, and Crgi45; Table I) demonstrate a large genetic divergence between samples from Santa

Table I. Estimation of divergence time (represented as the number of generations) between oyster samples based on the stepwise model of mutation of microsatellite markers. The analysis is based on the size (in base pairs) of the amplified alleles in oyster genotypes from Santa Catarina compared to alleles reported for the USA, Japan, and China and the number of generations required for the divergence to occur strictly following the stepwise mutation model (increase or decrease of one repeating motif in each generation).

Locus	Microsatellite motif	Santa Catarina[1]	USA[2]	Number of generations
ucdCg-170	$(GA)_n(GT)_n$	262-282	276	3-7
ucdCg-156	$(GATA)_n(GATG)_n(TA)_n$	162-182	235	13-36
ucdCg-199	$(CAT)_n$	171	270	24-49
ucdCg-152	$(CAT)_n$	272-282	257	5-8
Locus	Microsatellite motif	Santa Catarina[1]	Japan[3]	Number of generations
Crgi39	$(TC)_n$	252-270	190-214	22-30
Crgi4	(TTC)n $(CTTTT)_n$	152-182	235-238	1-30
Crgi45	$(AT)_n(ATTT)_n$	260	158-164	11-15
Locus	Microsatellite motif	Santa Catarina[1]	China[4,5]	Number of generations
ucdCg-170	$(GA)_n(GT)_n$	262-282	130-150	56-76
ucdCg-156	$(GATA)_n(GATG)_n(TA)_n$	162-182	220	9-29
ucdCg-199	$(CAT)_n$	171	260-270	22-49
ucdCg-152	$(CAT)_n$	272-282	270	1-4
Crgi39	$(TC)_n$	252-270	190-214	17-21
Crgi45	$(AT)_n(ATTT)_n$	260	158-180	6-25
Crgi4	(TTC)n $(CTTTT)_n$	152-182	235-255	3-9
otgfa00007B07	$(TA)_n$	272	279-295	2-14
otgfa00129E11	$(CA)_n$	182-192	155-185	4-19

[1]This study, [2]Li et al. (2003), [3]Sekino et al. (2003), [4]Qi et al. (2009),[5]Liu et al. (2017).

Catarina and from the USA, Japan, and China. This result suggests a large difference among the oyster samples compared in this study, potentially derived from the selection process (Figure 1d) and genetic improvement achieved in Santa Catarina.

The effect of selection and deep division of strains in the genetic improvement program was claimed by Zhang et al. (2023) to explain the evident genetic structure of the evaluated samples, even though the base population of each color strain was a subset of the original wild stock.

Based strictly on the SMM model, the divergence between the alleles observed in Santa Catarina samples and the samples from the USA, Japan, or China is the result of up to 76 mutational events. Focusing the analysis only on the alleles reported for the USA, two loci diverge between 13 and 49 generations (Table I). The selection of matrices aiming at the genetic improvement for the cultivation of oysters *C. gigas* in the North and South Bays in Santa Catarina Island started in 1987 with oysters imported from Chile. From the years 1996 to 2003 there were subsequent introductions of matrices from the USA. Considering the reproductive cycle of *C. gigas* in Brazil, from the time of the introduction of North American material up to 2022, it holds 38 to 52 generations of 6 months or 23 to 31 generations of 10 months. Congruently, Japanese samples (the origin of the North American samples) divergence from Santa Catarina samples ranges from 22 to 33 generations in locus Crgi39. Concerning oysters from China, this divergence reaches 49 generations in locus ucdCg-199 and up to 76 generations in locus ucdCg-170 (Table I).

In summary, this study demonstrates the effect of selection and reproductive isolation of Santa Catarina oysters on their allelic composition and genetic structure. There is a significant difference in the allelic composition of Santa Catarina oysters compared to oysters from other geographic origins, suggesting that the existing genetic structure is primarily due to these lineages' selection and reproductive isolation. Thus, the typical allelic patterns revealed in this study for the *C. gigas* cultivated in Santa Catarina Island support the need to protect this genetic resource and maintain the genetic improvement program for both commercial and scientific purposes. Moreover, these differences have been the basis of an ongoing project to structure a Geographical Indication on the Island of Santa Catarina and the surrounding municipalities: the Denomination of Origin "*Ostras de Floripa*" (Sebrae/SC, 2020), adding value to this product into the seafood market.

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VMS performed the sampling, genotyping, data analysis and wrote the first version of the manuscript. ADC contributed with financial support and manuscript writing.