


Are 45 years of reproductive isolation enough to prevent the amplification of mitochondrial genes in the Pacific oyster?

¿Son suficientes 45 años de aislamiento reproductivo para evitar la amplificación de genes mitocondriales en el ostión del Pacífico?

Felipe Reynaga-Franco¹, José Manuel Grijalva-Chon¹, Jorge Eduardo Chávez-Villalba², Reina Castro-Longoria¹, José Alfredo Arreola-Lizárraga², Ramón Héctor Barraza-Guardado¹

¹Universidad de Sonora, Departamento de Investigaciones Científicas y Tecnológicas. Hermosillo, Sonora, México.

²Centro de Investigaciones Biológicas del Noroeste. Guaymas, Sonora, México.

Correspondencia: José Manuel Grijalva-Chon  E-mail: manuel.grijalva@unison.mx

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The Pacific oyster culture in Mexico began 45 years ago, first with spat imported from the USA and now with spat produced in several local hatcheries. Oyster farmers do not know the parameters that define the quality of the spat they buy, among them the level of genetic variability available in the lots offered. In order to evaluate and compare the genetic variability in spat produced by four Mexican hatcheries, an attempt was made to amplify and sequence the non-coding region and the ND5 gene of the oyster mitochondrial DNA with oligos reported in the scientific literature. The amplification of the non-coding region was not possible due to the bad design of the oligos. Despite the integrity of the extracted oyster DNA, the ND5 gene was not able to be amplified possibly due to the modification of the oligo recognition site in the gene. The generational separation of the oyster cultivated in Mexico from its original source population makes it necessary to obtain new mitochondrial sequences in order to design new oligos suitable for the populations established in Mexico.

Palabras clave

Crassostrea gigas,
Acuicultura,
Ostricultura,
ADN mitocondrial,
Genética de
poblaciones.

RESUMEN | El cultivo del ostión del Pacífico en México inició hace 45 años, primero con semilla importada de los EUA y ahora con semilla producida en laboratorios locales. Los ostricultores no conocen los parámetros que definen la calidad de la semilla que ellos compran, como el nivel de la variabilidad genética disponible en los lotes ofertados. Con el fin de evaluar y comparar la variabilidad genética de la semilla producida en cuatro laboratorios mexicanos, se hizo un intento de amplificar y secuenciar la región no codificante y el gen ND5 mitocondriales del ostión con oligos reportados en la literatura científica. La amplificación de la región no codificante no fue posible debido al mal diseño de los oligos. A pesar de la integridad del ADN extraído de los ostiones, el gen ND5 no pudo ser amplificado debido posiblemente a la modificación del sitio de reconocimiento del oligo en ese gen. La separación generacional del ostión cultivado en México de su población fuente original hace necesario obtener nuevas secuencias mitocondriales para diseñar nuevos oligos adecuados a las poblaciones establecidas en México.

The Pacific oyster, *Crassostrea gigas*, was introduced approximately 45 years ago in Mexico with the purpose of diversifying the aquaculture and fishing activities in the country's Pacific Ocean coast (Islas-Olivares, 1975; De la Rosa-Vélez *et al.*, 1991; Chávez-Villalba, 2014). Since then, it has supported small and medium producers that supply the national market, although they are far from reaching the production level of shrimp farming on the same Mexican coast. Based on molecular data, Salvi *et al.* (2014) and Salvi & Mariottini (2017) have proposed that the genus *Crassostrea* of the Indo-Pacific cupped oysters be changed to *Magallana*, and that *Crassostrea* remain in the Atlantic cupped oyster species. Although the proposal has been accepted by the World Register of Marine Species, there is strong controversy in this regard, so Bayne *et al.* (2017) and Backeljau (2018) recommend continuing with the denomination *Crassostrea gigas*, which is taken into account in this work. The Mexican oyster culture in its beginnings imported the spat from the USA, first of diploid organisms and later also of triploid, depending on their availability. Currently, there are several Mexican oyster hatcheries that supply spat, mainly diploid, to

oyster farms. These laboratories are often supplied with broodstock from the same farms to whom they sell the spat, but occasionally they obtain breeders from South America or Europe.

In species of interest for aquaculture and that are subject to a closed-cycle culture, with total control of reproduction and progeny, genetic variability is a very important factor that contributes to the quality of the species offered to the aquaculturist. This variability may reflect the effects of prolonged artificial selection, cumulative inbreeding, divergence from the original source and bottlenecks, since all of the above leads to a loss of the potential for adaptability in populations (Barg, 1992). Studies on the structure and population genetic variability of the Pacific oyster have been carried out with allozymes, microsatellites, AFLP, and mitochondrial DNA sequences in natural (Ozaki & Fujio, 1985; Yang *et al.*, 2000; Yu *et al.*, 2008), feral (Li *et al.*, 2003; Meistertzheim *et al.*, 2013) and cultured populations (De la Rosa-Vélez *et al.*, 1991; Launey & Hedgecock, 2001, Li *et al.*, 2006; Enríquez-Espinoza & Grijalva-Chon, 2010). In the study of mitochondrial variability, the non-coding control region has been widely used for analysis of polymorphism and population variability (Ferris & Berg, 1987; Allendorf *et al.*, 2013). However, recently the mitochondrial NADH dehydrogenase 5 (ND5) gene has been used successfully for the analysis of the population genetic structure (Makhawi *et al.*, 2013), even in the Pacific oyster (Kawamura *et al.*, 2017). In the case of *C. gigas* cultivated in Mexico, little has been studied, excepting some research using allozymes (De la Rosa-Vélez *et al.*, 1991; Correa *et al.*, 2004; Enríquez-Espinoza *et al.*, 2010), and microsatellites (Cruz *et al.*, 2007; Grijalva-Chon *et al.*, 2013). In order to compare the differences in the genetic variability of the oyster spat that occurs in northwestern Mexico, we aimed to conduct a study of population genetics in the spat produced by the four largest commercial hatcheries in the region.

In May 2014, four lots of 10,000 spat each were received from the four main oyster hatcheries in northwestern Mexico. The spat were grown in Nestier modules distributed in long-line in the La Cruz coastal lagoon, Sonora, Mexico (Gulf of California, 28° 47' 34" N, 111° 53' 27" W, see Reynaga-Franco *et al.* (2019) for details about hatcheries and geographical location). After three months of culture, 50 organisms per lot were taken, the shell was removed with the help of dissection forceps, and the soft tissue was fixed in 1.5 ml tubes with absolute ethyl alcohol (> 95%) and transferred to the Molecular Ecology Laboratory of the University of Sonora to be processed. Subsequently, a 20-25 mg portion of the tissue preserved was cut and the DNA extraction was performed using the QIAamp DNA Mini Kit according to the manufacturer's specifications (QIAGEN). The concentration (ng/μl) and purity (A_{260}/A_{280}) was estimated with a Nanodrop 1000 spectrophotometer.

To corroborate the integrity of the DNA extract as well as the absence of inhibitory factors, a PCR amplification was performed with the universal oligos CAS1S: 5'-GGAATTGACGGAAGGGCACC-3' and CAS2: 5'-ACGGGCGGTGTGTACAAAGG-3', reported by Le Roux *et al.* (1999). These oligos amplify a 521 bp section of the 18S rDNA nuclear gene from a large number of eukaryotic species, including mollusks. To evaluate the genetic variability of *C. gigas*, the mitochondrial non-coding region and the mitochondrial NADH subunit 5 gene (ND5) were selected. For an expected amplicon of 739 pb of the non-coding region of mitochondrial DNA, oligos 5'-TCACAAGTACATTTGTCTTCCA-3' and 5'-AACGTTGTAAGCGTCATGTAAT-3' reported by Aranishi & Okimoto (2005) were used. For the ND5 gene, the oligos CgND5.6 F5: 5'-TAGCCCTACAGTTTCATTATCG-3' and CgND5.6 R5: 5'-AAATACTTACAGGAAAAGCGA-3', reported by Kawamura *et al.* (2017) were used to amplify a 524 bp fragment. All PCR reactions were carried out with lyophilized PuRe Taq Ready-to-Go PCR beads (GE Healthcare), following the proportions and PCR conditions recommended by the above mentioned authors. Additionally, we conducted some tests modifying the conditions reported by the authors of the oligos used in this study. First, we modified the thermal cycling times of the denaturation, alignment and extension steps reported by Aranishi & Okimoto (2005), since they reported a few seconds for each step and we increased to 60 seconds for each step. In the case of the oligos from the non-coding region and of ND5, gradient PCR of both alignment temperature (from 51 to 61 °C for non-coding region oligos, and from 50 to 59 °C for ND5 oligos) and concentration of MgCl₂ (from 1.5 to 5.0 mM) were also performed. DNA amplifications were carried out in a BioRad DNA Engine thermal cycler, and a replica in a subset of 10 organisms per lot was carried out in a Techne TC-412 thermal cycler. The electrophoresis was on 2% agarose gels and 15 V/cm for 40 minutes to observe the results of the amplification.

The average of the A_{260}/A_{280} ratio was 2.07 ± 0.2 , considering the 200 samples, which indicates acceptable values of purity. However, it was not possible to obtain amplicons of the two mitochondrial regions in any of the 200 samples, neither with the original conditions reported by the authors, nor with the change of the thermal cycling parameters, nor changing the thermal cyclers. However, because the universal oligos corroborated the absence of inhibitory factors, integrity and good quality of the oyster DNA extract (Fig. 1), an analysis of the design quality of the oligos was carried out using the Multiple Primer Analyzer software available online at www.thermofisher.com. This analysis showed that the oligos for ND5 are well designed, but those reported by Arinichi & Okimoto (2005) form cross dimers (Fig. 2), being unusable for the amplification of the oyster DNA.

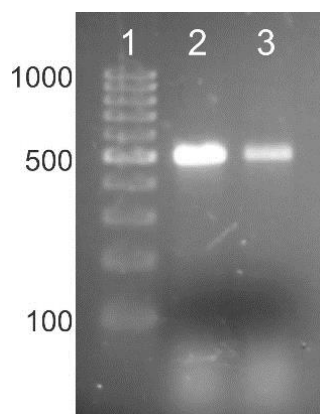


Figure 1. PCR amplification using universal oligos CAS1S/CAS2. Line 1: 100 bp DNA ladder. Line 2 - 3: amplicon of 521 pb of two *Crassostrea gigas* samples.



Figure 2. Cross primer dimer obtained with oligos reported by Arinichi and Okimoto (2005) for *Crassostrea gigas* mitochondrial non-coding region.

The use of oligos reported in the scientific literature saves time, money and effort because they represent the validated effort of a research group. However, there are cases of extended use of oligos where years later it is shown that they result in a high frequency of false negatives (Lynch *et al.* 2013). Surprisingly, considering the elapsed time, we did not find scientific literature that reported the use of the oligos of Arinichi & Okimoto (2005), nor other studies that report having failed to replicate the findings of these authors. Considering that the universal oligos gave favorable results, and the bad design of the oligos reported by Arinichi & Okimoto (2005), it can be considered that the cause of the negative amplifications of ND5 is the variation in the target sequences in the oysters. If we consider that mitochondrial DNA has a high evolutionary rate, 5 - 10 times faster than nuclear DNA (Brown *et al.*, 1979), and due to the time elapsed and the isolation of populations of Pacific oysters cultivated in Mexico from wild populations in Japan, there may now be a degree of genetic differentiation that prevents PCR amplification using the oligonucleotides reported by Kawamura *et al.* (2017) due to point mutations accumulated in the oyster recognition sequence. However, to get an idea of the magnitude of the divergence between the oyster isolated and cultivated in the American continent and the natural populations of the western Pacific, it is necessary to look for a suitable mitochondrial genetic marker, which would be interesting to address in the short term.

The use of DNA microsatellites is a good alternative to study the population genetic structure in *C. gigas* (Magoulas *et al.*, 1998; Huvet *et al.*, 2000; McGoldbrick *et al.*, 2000; Launey & Hedgecock, 2001; Li *et al.*, 2003; Li *et al.*, 2006; Yu *et al.*, 2008; Grijalva-Chon *et al.*, 2013), but the analysis of mitochondrial gene sequences is a methodology that is cheaper since it requires less time in laboratory and in the data

analysis. For this reason, it is necessary to design new oligos for the genetic analysis of the mtDNA of the Pacific oyster cultivated in the American continent, and particularly in Mexico. However, although there is information of mitogenomes of *Crassostrea* (Yu & Li, 2012), there is no information on mitochondrial sequences of organisms grown in America so that new oligos can be designed from them. This is a need that must be addressed and resolved in the short term.

The analysis of the genetic variability of oyster spat produced in northwestern Mexico is one of the necessary elements to evaluate the quality of the spat offered, since a fundamental part of this attribute comes from the breeders, which are selected by their physical attributes and not because of their genetic attributes. As a measure of the diversification of genetic variability, some hatcheries exchange breeders, occasionally with the importation of a lot from abroad (Grijalva-Chon *et al.*, 2013), but no one has imported organisms from Japan or its adjacent waters.

From the perspective of the hatchery owners, it is not very profitable to pay the costs of a possible genetic monitoring in each lot produced, or even once a year. Doing this would make the necessary adjustments to maintain a stable genetic variability and would obtain a spat with better resistance to pathogens, better growth rates and better biological performance in general (Dégremont *et al.*, 2007). In addition, to reinforce the spat quality, a strict follow-up of the pedigree would have to be implemented to maintain an acceptable level of heterozygosity and minimal inbreeding. The relevance of genetic variability in oyster spat is undeniable, so establishing the criteria to evaluate it and to include it in the spat production protocols is the main challenge in this field for oyster hatcheries in the world.

In aquaculture, assessing the genetic variability of selected strains in oyster hatcheries provides information that may be related to the biological performance of cultured organisms. In this way, a strain with a good level of genetic variability will surely generate good yield at the time of harvest. In the case of the Pacific oyster strains studied here, there is no knowledge of the level of genetic variability associated with them. The mutational rate of the mitochondrial genome is much higher than that of the nuclear genome, and considering that the Pacific oyster cultivated in Mexico has more than 40 years of isolation from its native population, it is necessary to obtain the sequence of the non-coding mitochondrial region of the Mexican oyster strains in order to design new oligos that allow using this section of the genome to make estimates of genetic variability.

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