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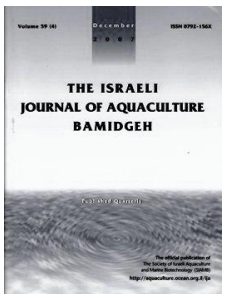

An Improved Method of DNA Extraction from the Shell of the Pacific Oyster, *Crassostrea gigas*

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Key words: mollusc, bivalve, Pacific oyster, shell DNA, marker-assisted selection (MAS)

Abstract

We earlier developed a method to extract DNA from *in vivo*-sampled mantle tissue of the bivalve Pacific oyster (*Crassostrea gigas*) for molecular marker-assisted selection (MAS) in breeding. However, mortality was too high when using this method. In the current study, a more efficient and safer method of extracting DNA from the oyster shell was developed, based on the improved phenol-chloroform DNA extraction method. Both nuclear DNA and mitochondrial DNA were successfully extracted from the oyster shell, and mortality was 0%. These results illustrate the safety of extracting DNA from the bivalve shell. In addition, because DNA is a cellular component, the results provide molecular evidence that cells are likely involved in shell formation.

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Introduction

Molecular marker-assisted selection (MAS) has been successfully used in domesticated land-dwelling animals (Villanueva et al., 2005; Uemoto et al., 2011), partly because samples can be expediently acquired and prepared for DNA extraction and further molecular analysis without killing the animal. In contrast, MAS of aquatic animals, such as commercially important bivalves, has proven difficult. Obtaining tissue samples from the soft body without affecting the animal's growth and survival is difficult, and this problem has restricted the application of MAS in bivalve breeding. We earlier developed a genomic DNA extraction technique that involved *in vivo* sampling of mantle tissue of the Pacific oyster (*Crassostrea gigas*), but the method caused considerable mortality - 16.7% (Wang et al., 2011). In the current study we developed a safer method based on the phenol-chloroform DNA extraction method (Sambrook and Russell, 2001) that involves extracting DNA from the Pacific oyster shell.

Materials and Methods

Shell DNA extraction and mortality analysis. Forty wild Pacific oysters from a single population were bought from Nanshan Market (Qingdao, China) and cultivated in our laboratory. The shell of each oyster was cleaned with a brush, and a piece of the left shell (the bigger shell; about 1 cm^2 and 1 g) was sheared off from the ventral edge. Thirty of the oysters were used to test for mortality after the shell piece was removed. After one month, mortality was calculated based on the number of dead individuals.

The other 10 oysters were used for DNA extraction. The sheared-off shell pieces were cleaned with deionized water and placed in an oven for 6 h at 65°C until completely dry. The periostraca of the shell pieces were removed using abrasive paper to prevent contamination with DNA from other organisms. The shell piece from each oyster was triturated in a mortar, transferred to ten 1.5-ml EP tubes at 100 mg/tube, and 1 ml EDTA (pH = 8.0) was poured into each tube. The tubes were shaken at 200 rpm for 24 h at 37°C on a shaker to induce decalcification. The tubes were centrifuged at 1538 × *g* for 15 min, the supernatant fluids were discarded to remove redundant saline ions, and the sediment was washed with 50 µl deionized water. Next, 600 µl Tris-Hcl and 20 U proteinase K were added to the tubes and the contents were incubated for 6 h at 56°C.

In the next step, 600 µl PCI (phenol:chloroform:isoamyl alcohol = 25:24:1; vol/vol) was added to each tube. The solution was mixed and centrifuged at 13,845 × *g* for 15 min. The supernatant fluids were transferred to new 1.5-ml EP tubes and the step was repeated. Into each tube, 600 ml chloroform was added, and the solution was mixed and centrifuged at 13,845 × *g* for 10 min. The supernatants were again transferred to new 1.5-ml tubes to which 600 ml isoamyl alcohol (precooled at -20°C) was added. After complete mixing and centrifugation at 13,845 × *g* for 10 min, the supernatants were discarded. The sediments in each tube were washed twice with 50 µl 75% ethanol, air dried, and dissolved in 5 µl deionized water. The DNA solutions in all 10 tubes were combined, and the total volume was 50 µl for each of the 10 oysters.

As a control, the mantle from each oyster was sampled and DNA was extracted using the usual phenol-chloroform method (Sambrook and Russell, 2001).

Species identification using PCR. To confirm that the DNA extracted from the oyster shells was not contaminated by other organisms inhabiting the shell, fragments of the cytochrome oxidase subunit 1 (CO1) and beta-actin genes were amplified, sequenced, and aligned with sequences in the nucleotide (Nt) database at the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi). PCR was performed in a volume of 25 µl that contained 5 × PCR buffer, 0.2 mM deoxynucleotide triphosphate (dNTP), 2.5 mM $MgCl_2$, 0.5 U Taq DNA polymerase, and 0.5 mM of each primer (Table 1). PCR conditions were: 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 20 s, and a final extension at 72°C for 5 min. The PCR products were run on 1.2% agarose gel in 1 × TBE buffer to determine their molecular weight and sequenced in Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., to obtain nucleotide sequences.

Table 1. Primers used to amplify segments of the cytochrome oxidase subunit 1 (CO1) and beta-actin genes of pieces of Pacific oyster shell.

CO1-F[1]	GGTCAACAAATCATAAAGATATTGG
CO1-ari[2]	AAAAAAGATTATAACTAATGCATGTCGG
CO1-ang[2]	AGTTACCAAACCCCCCAATTATCAGG
CO1-gig[2]	TCGAGGAAATTGCATGTCTGCTACAA
CO1-hk[2]	GGAGTAAGTGGATAAGGGTGGATAG
CO1-sik[2]	AAGTAACCTTAATAGATCAGGGAACC
Beta actin-F[3]	CCGACGGTCAGGTCATCAC
Beta actin-R[3]	CTCATCGTACTCCTGCTTG

[1] the universal forward primer for the CO1 gene
[2] Five kinds of reverse primers for the CO1 gene: CO1-ari specific to *Crassostrea ariakensis*; CO1-ang specific to *C. angulata*; CO1-gig specific to *C. gigas*; CO1-hk specific to *C. hongkongensis*; CO1-sik specific to *C. sikamea*
[3] The forward and reverse primers for the beta-actin gene.

DNA productivity calculation. DNA yield (ng) was calculated as: (A_{260} reading - A_{320} reading) × dilution factor × 50 ng/µl × total sample volume in µl. DNA yield per microgram shell (i.e., productivity; ng/mg) was calculated as: DNA yield/shell wt.

Results

After one month, all 30 oysters with part of their shell removed were alive, indicating that the sampling procedure did not harm the oysters.

Four electrophoretic bands for total DNA appeared on the agarose gel, although they were not very distinct (Fig. 1a), suggesting that DNA can be extracted from oyster shells but that productivity is not high (0.0202 ng/mg). When CO1-F and CO1-gig were used as the PCR primers, CO1 gene fragments were detected using agarose gel electrophoresis (Fig. 1b); the similar electrophoretic mobility of the bands in lanes 1 (mantle DNA) and 2 (shell DNA) suggests that the molecular weights of the amplified CO1 fragments from the mantle and shell are identical. When aligning the nucleotide sequences of the amplified shell DNA segments with the Nt database, the first hit gene was the CO1 gene of *C. gigas* (Fig. 2). Fragments of the beta-actin gene were amplified and detected using the same methods (Fig. 1c); the fragment size and BLAST results also suggest that the shell DNA belongs to *C. gigas*.

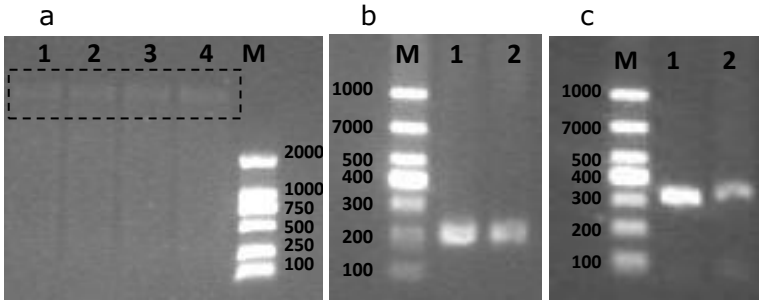

Fig. 1. Agarose gel electrophoresis of total DNA extracted from Pacific oyster shell and PCR products of the CO1 and beta-actin genes: (a) total DNA extracted from the oyster shell is shown in the dashed rectangle, (b) lane 1 = PCR products of the CO1 gene using mantle DNA as the template, lane 2 = PCR products of the CO1 gene using shell DNA as the template, and (c) lane 1 = PCR products of the beta-actin gene using mantle DNA as the template, lane 2 = PCR products of the beta-actin gene using shell DNA as the template.

a

Accession	Description	Max score	Total score
HQ718599.1	Crassostrea gigas voucher IOCAS:JZ crgi2 cytochrome oxidase subunit	486	486
HQ718598.1	Crassostrea gigas voucher IOCAS:JZ crgi1 cytochrome oxidase subunit	486	486
AF280608.1	Crassostrea gigas cytochrome oxidase subunit I gene, partial cds; mito	486	486
AF177226.1	Crassostrea gigas mitochondrial DNA, complete genome	468	468
HM626170.1	Crassostrea gigas isolate 625 cytochrome c oxidase subunit I (COI) ger	464	464

b

Accession	Description	Max score	Total score
AB071191.1	Crassostrea gigas actin 2 mRNA for Actin 2, complete cds	675	675
U55046.1	Placopecten magellanicus actin mRNA, complete cds	525	525
DQ787858.1	Mizuhopecten yessoensis beta-actin mRNA, complete cds	497	497
GU263793.1	Haliotis midae clone Hdd.c2 microsatellite sequence	486	486
AY380809.1	Haliotis discus hannai actin mRNA, complete cds	486	486

Fig. 2. BLAST results of amplified (a) CO1 and (b) beta-actin fragments of shell DNA from Pacific oysters.

Discussion

While organisms could have parasitized and contaminated the oyster shell, the results presented in Fig. 1 and the BLAST results suggest that the DNA extracted from the oyster shell belonged to *C. gigas* and not to other organisms.

The CO1 gene is a mitochondrial gene (Patil et al., 2005), the beta-actin gene is a nuclear gene, and DNA is a component of cells. The coexistence of mitochondrial and nuclear DNA in the oyster shell suggests that cellular components are embedded in the shell. The classic opinion on shell formation emphasizes that organic elements in the shell are secreted by the mantle and that cells are not directly involved in shell formation (Furuhashi et al., 2009). However, another hypothesis posits that one type of cell, the hemocyte, can be directly involved in shell biomineralization in the eastern oyster (Mount et al., 2004). Our observation of the presence of a cellular component (DNA) in the oyster shell supports the latter opinion.

In contrast to sampling the mantle, shell sampling did not damage the oyster's soft body, and mortality was significantly lower (16.7% after sampling the mantle and 0% in the current study). Thus, extracting DNA from the oyster shell is safer than extracting it from the mantle. This new technique should accelerate the application of MAS in bivalve shellfish breeding.

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