

Simple and chemical DNA extraction from preserved bivalve mantle for reproducible PCR amplification

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Abstract DNA-based studies have been one of the major interests in phylogenetic structure analysis for both wild populations and cultured broodstocks of commercially important marine bivalves. A simple and reliable method for extracting genomic DNA from preserved mantle tissues of Pacific oyster *Crassostrea gigas* was developed for reproducible PCR amplification. The methodology was based on destruction of the tissue using Proteinase K, Chelex 100 resin, detergents and urea, followed by preferential capture of DNA using silica particles. Approximately 5 mg of the tissue provided the quality and quantity of DNA enough for several hundreds of PCR reactions amplifying the hypervariable mitochondrial DNA intergenic spacer of Pacific oyster. Since being applicable for DNA isolation from not only fresh and frozen but also ethanol-preserved mantle tissues, this rapid and economical method can serve for investigating a large number of field bivalve specimens transported in ethanol at the ambient temperature from local collection sites.

Key words Marine bivalves, mantle, DNA extraction, PCR amplification.

Introduction Recent studies on phylogenetic structure analysis rely increasingly on DNA-based techniques by means of PCR amplification of polymorphic genetic markers for detecting genetic divergences within and among populations. However, the feasibility of these techniques is sometimes limited by the ability to prepare genomic DNA of acceptable quality and quantity from collected tissues of specimens. Adductor muscle of bivalves is a reliable source of DNA from aquaculture species, such as oyster, clam, and scallop. For the health assessment of bivalves, adductor muscle is also a reliable source of storage glycogen that is a biomarker highly sensitive to physiological changes and environmental alterations including pollutants. Alternative tissues are therefore required as a DNA source, particularly when both phylogenetic and physiological analyses are performed on the same specimens of bivalves. This study developed a simple and reliable method for extraction of genomic DNA from Pacific oyster *Crassostrea gigas* mantle for PCR amplification of the hypervariable mtDNA noncoding region, which is a useful genetic marker for population structure analysis (1). Since being applicable for DNA preparation from not only fresh and frozen but also ethanol-preserved samples, this rapid and economical method can serve for investigating a large number of field specimens that are transported in ethanol from local collection sites.

Methods Mantle tissues were collected from fresh adult oysters and ethanol-preserved adult oysters transported at the ambient temperature from local collection sites in and out of Japan. They were then either stored at -20°C or preserved in ethanol at the ambient temperature until DNA extraction. Total genomic DNA was extracted from fresh, frozen and ethanol-preserved mantle tissues. Approximately 5 mg of the tissues (3–7 mm²) were placed into 1.5-ml Eppendorf tubes containing 0.1 ml of 4 M urea, 1 % Tween 20, 1 % Nonidet P-40, 5 % Chelex 100 and 0.005 mg Proteinase K in sterile water. After thoroughly mixing by vortex, the tubes were incubated at 55 °C for 30–60 min, and then boiled for 8 min. Following centrifugation at 15,000 g for 5 min, the precipitate containing the chelating resin and cell debris was discarded. Approximately 0.1 ml of the supernatant containing DNA was collected and adjusted pH at 8.0, and then divided into two portions. To a portion of 0.05 ml of the DNA solution, 0.025 ml of 7.5 M ACNH₃ and 0.2 ml of ethanol were added and mixed by vortex. Following centrifugation at 15,000 g for 20 min, the DNA pellet was washed thrice with ethanol, dried, and resuspended in 0.1 ml of 10T0.1E (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, pH 8.0). To the other portion of 0.05 ml of the DNA solution, 0.05 ml of 6 M KI and 0.025 ml of silica particles (2) were added and mixed by vortex. Following centrifugation at 2,000 g for 1 min, the silica pellet was washed twice with STE buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 50 % ethanol) and then dried. DNA bound to the silica particles was eluted with 0.1 ml of 10T0.1E by incubation at 65 °C for 10 min followed by centrifugation at 2,000 g for 1 min.

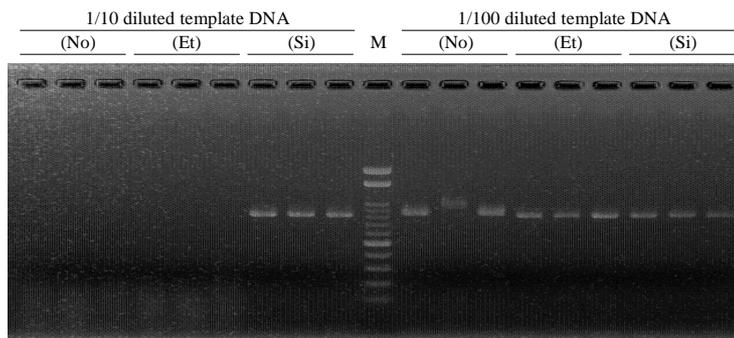


Figure 1. PCR amplification of a 818-bp mtDNA fragment using genomic DNA prepared from ethanol-preserved oyster mantle with different procedures; extraction using Proteinase K, Chelex 100, detergents and urea (No) and subsequent ethanol precipitation (Et) or silica treatment (Si). lane M, 100 bp ladder plus.

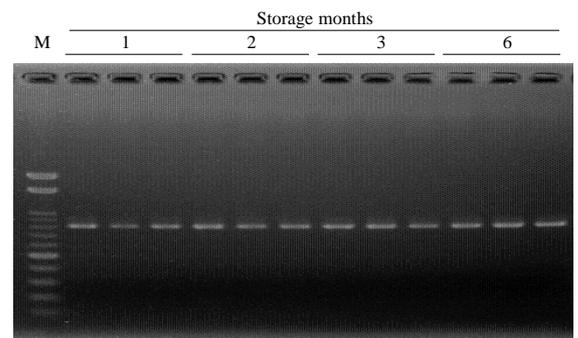


Figure 2. PCR amplification of a 818 bp fragment using 1–6 months-storage genomic DNA prepared from ethanol-preserved oyster mantle by extraction using Proteinase K, Chelex 100, detergents and urea and subsequent silica treatment. lane M, 100 bp ladder plus.

Results From 9.6–13.8 g of a shelled oyster, two pieces of 491.5–808.5 mg of the mantle tissue (76.4–90.6 mm in length; 10.5–13.1 mm in width) could be collected, and this corresponded to 0.72–1.70 mg/mm². A small tissue portion (3–7 mm²) weighing approximately 5 mg, provided the quality and quantity of DNA enough for over 1,000 PCR reactions amplifying a 818 bp fragment involving the hypervariable mtDNA noncoding region of Pacific oyster (1). The methodology was based on [1] destruction of the mantle tissue using Proteinase K, Chelex 100 chelating resin, mixed detergents, and a high concentration of urea, [2] followed by preferential capture of DNA using silica particles and removal of other tissue components such as protein and cell debris. Both steps proved to be essential for obtaining genomic DNA of good quality and relatively large quantity (Figure 1). As documented in many previous reports, mechanical tissue homogenization can be an efficient step to extract significant amounts of DNA. However, we employed a high concentration of urea; 4 M was enough, and it worked well as an alternative to mechanical tissue homogenization. Urea treatment was thus an efficient step to breakdown connective tissue matrices of oyster mantle by protein denaturation. We compared the purification methods of the extract obtained from the step [1], and silica treatment was verified to be more effective and reproducible in PCR amplification regardless of the concentrations of DNA, when compared to ethanol precipitation (Figure 1). This result suggests the inability of ethanol precipitation to completely remove PCR-inhibiting materials present in the mantle tissue. In addition, silica treatment was useful to separate polysaccharides from DNA (2). As bivalves have the nature of rich polysaccharides, silica treatment seems to be the optimal method to isolate genomic DNA from the tissue extracts of not only mantle but also other tissues of bivalves.

Discussion Genomic DNA preparation for PCR amplification has become one of the major concerns in molecular-based phylogenetic analysis for wild populations and cultured broodstocks of commercially important bivalves, especially when a large number of samples must be processed. From ark shell and scallop (3) and larvae and juveniles of oyster (4), genomic DNA was extracted using 5 % Chelex 100 resin combined with Proteinase K digestion, and then directly applied to PCR amplification with no further purification. In our experiments, however, by using the extract obtained from the step [1] with neither subsequent ethanol precipitation nor silica treatment, no reproducible amplification was detected (Figure 1). Presumably, this was due to significant amounts of protein and other cell materials present in the mantle tissue, which might inhibit PCR amplification. In diagnostic study for detection of the oyster parasite, similar attempt was made to extract DNA from adult oyster mantle by the standard Chelex 100 resin method (5). They selected the mantle tissue and Chelex method, because it was described to be easy collection of the tissue after opening the shells, sufficient DNA for analysis contained in a small tissue portion, and simple and economy as compared with other conventional DNA extraction methods. Although they reported to successfully perform PCR amplification using oyster DNA obtained by the standard Chelex method, which almost corresponded to the step [1] in our protocol, we detected no amplification (Figure 1). This might be due to either different conditions of sacrificed oyster specimens or different techniques for tissue destruction between chemically and mechanically. Our results demonstrate that the combined method for a simple extraction using the urea-Chelex-Proteinase K buffer with subsequent silica treatment can be useful and reliable for DNA preparation from not only fresh and frozen but also long-term preserved oyster mantle tissues. In addition, genomic DNA prepared according to this method was highly stable for up to 6 months evidenced by successful PCR amplification (Figure 2). The procedure is quite simple, reliable, and economical, and requires neither hazardous reagents nor expensive equipments. It therefore can serve for investigating a large number of field bivalve samples that are preserved in ethanol and next transported at the ambient temperature from local collection sites worldwide.

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