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Rapid isolation of genomic DNA from Asian green-lipped mussel (*Perna viridis*) for random amplified microsatellite polymorphism

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Abstract: The objective of the present study was to develop a rapid, reliable and yet inexpensive protocol for genomic DNA extraction from frozen and ethanol-preserved Asian green-lipped mussels for random amplified microsatelite (RAM) analysis. The procedure comprised of three major steps: (1) Tissue degradation by boiling in 6% Chelex 100 resin in TE buffer; (2) Protein digestion by Proteinase K; and (3) DNA precipitation by adding 2 volumes of cold absolute ethanol. The entire procedure can be completed within two hours. The resulting RAM profiles were clear and reproducible. Our results demonstrate that the combined protocol of Chelex 100-Proteinase K-ethanol precipitation is a powerful yet economical DNA isolation method for population genetic studies involving a large sample size.

Keywords: DNA extraction, mussel, Chelex 100 resin, RAM

Introduction

The Asian green-lipped mussel, *Perna viridis* (L.) (Class Bivalvia: Family Mytilidae), is widely distributed in the Indo-Pacific region (Siddall, 1980). It can be found along the west and east coasts of Peninsular Malaysia and also in certain areas of Sabah. It is an economical source of animal protein for human consumption. Today, it is being extensively cultured in many Asian countries including Malaysia (Rosell, 1991; Monirith *et al*., 2003). Due to its widespread distribution in this region, this species has been an important bioindicator of a wide range of heavy metal contaminants in the marine environments (Nicholson and Lam, 2005; Lily *et al*., 2005). A number of studies have been carried out to investigate the population genetics and also in improving the broodstocks of this commercially important seafood delicacy and hence increase the productivity of mussel hatchery (Yap *et al*., 2002; Yap *et al*., 2004; Lily *et al*., 2005).

Recently, polymerase chain reaction (PCR) method has been widely applied in modern studies of population genetics for detecting genetic diversity within and among populations. However, the feasibility of such studies is always limited by the lengthy and labour-intensive procedure of DNA isolation from the species. Realizing the need for a rapid and simple procedure in DNA isolation from marine species particularly in bivalve, a number of studies have reported on the development of a rapid procedure for DNA extraction (Banerjee *et al*., 1995; Estoup *et al*., 1996; Nelson *et al*., 1998; Taris *et al*., 2005; Aranishi and Okimoto, 2006). However, a majority of the rapid methods were developed for PCR detection of certain genes which were not always suitable and applicable for molecular typing.

Here, we developed a rapid, simple and inexpensive method for DNA isolation from the mantle of Asian green-lipped mussel (*Perna viridis*) for PCR amplification of random amplified microsatellite (RAM). The DNA isolation procedure is applicable for DNA extraction from frozen and ethanol-preserved mussel, and is a suitable procedure for phylogenetic studies involving a large number of specimens in either frozen or ethanol-preserved form.

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Materials and methods

Asian green-lipped mussels

Asian green-lipped mussels were collected from a commercial mussel hatchery situated along the west coast of Peninsular Malaysia. The mussels were washed to remove the mud and the mantles were separated from the mussels. Half portion of the mantle tissue was packed into an individual bag and was kept at -20°C; another half was immersed in absolute ethanol and kept at 4°C. All processed specimens were kept for several days before DNA extraction.

DNA extraction

Approximately 25 mg of fresh or ethanol-preserved mussel mantle tissues were weighted into sterile 2 ml microcentrifuge tubes containing 600 ul of Chelex 100 resin (Sigma) in TE buffer (10 mM Tris-HCl, pH 7.5; 0.1 mM of EDTA, pH8.0). The tubes were boiled in a water bath for 10 min, and then they were cooled down to 55°C. Thirty microliters of 20 mg/ml Proteinase K (Sigma) was added to each tube and followed by incubation at 55°C for 1 hour. The tubes were mixed by gently flicking every 15 min. After that, the tubes were again boiled for 5 min. Following centrifugation at 10 000 g for 5 min, the precipitate containing Chelex 100 resin and cell debris was discarded. One hundred microliters of the supernatant containing DNA was transferred into three individual 1.5 ml tubes each. The first tube containing 180 µl of DNA lysate was immediately kept in 4°C for PCR amplification while the other two proceeded with DNA purification steps. A volume of 540 µl of 6 M NaI (Sigma) and 10 µl of 100% (wt/vol) silica (Yue and Orban, 2001) were added into one of the tubes with 180 µl of DNA lysate. The tube was vortexed briefly then shaken gently for 1 min and centrifuged at 10 000 g for 3-5 sec. The supernatant was discarded and the pellet was washed with 1 ml of wash solution (10 mM Tris, pH 7.5; 1 mM EDTA, pH 7.5; 100 mM NaCl, and 50% ethanol). The tube was centrifuged at 10 000 g for 10 sec and the silica-bound genomic DNA was dried at 37°C for 15 min. The DNA was then eluted by adding 180 µl of TE buffer and centrifuged at 10 000 g for 1 min. The supernatant containing genomic DNA was transferred into new tubes. The remaining tube with 180 µl of supernatant proceeded with DNA precipitation by absolute ethanol. A volume of 9 µl of 5 M NaCl was added to the DNA lysate and followed by 2 volumes of cold absolute ethanol. The mixture was mixed by gently inverting the tube and then kept at -20°C for 5 min. After that, centrifugation at 10 000 g was carried out for 5 min to pellet the genomic DNA in the solution. The DNA pellet was dried at 37°C for 10 min and resuspended with 180 µl of sterile TE buffer.

Table 1. Purity (A_{260}/A_{280}) and Yield (ng) of genomic DNA obtained from frozen and ethanol-preserved green-lipped mussels (*Perna viridis*) mantle by using various combinations of procedures

Chelex 100 resin		✔	✔	✔	✔	✔	
Proteinase K		✔	✔	✔			✔
Absolute ethanol			✔			✔	✔
Silica				✔			
Purity[a]	**Frozen**	1.22 (0.09)	1.71 (0.08)	1.79 (0.01)	1.48 (0.16)	1.70 (0.08)	1.69 (0.20)
	Ethanol-preserved	1.37 (0.05)	1.85 (0.08)	1.92 (0.04)	1.54 (0.12)	1.72 (0.02)	1.61 (0.09)
Yield[b] (ng)	**Frozen**	45.30 (2.00)	13.70 (1.47)	8.67 (1.45)	6.47 (0.76)	5.43 (0.61)	11.10 (1.20)
	Ethanol-preserved	43.70 (1.30)	12.60 (1.60)	9.40 (0.56)	6.37 (1.29)	5.13 (0.23)	10.60 (1.31)

[a] Purity of DNA is expressed in ratio of A_{260}/A_{280}

[b] Total yield of DNA is calculated by using formula: (A_{260} - A_{320}) x 50 (DNA extinction coefficient) x dilution factor x final sample volume; and converted to nanogram scale by multiply by 1000

[c] All data are recorded as: mean (standard deviation)

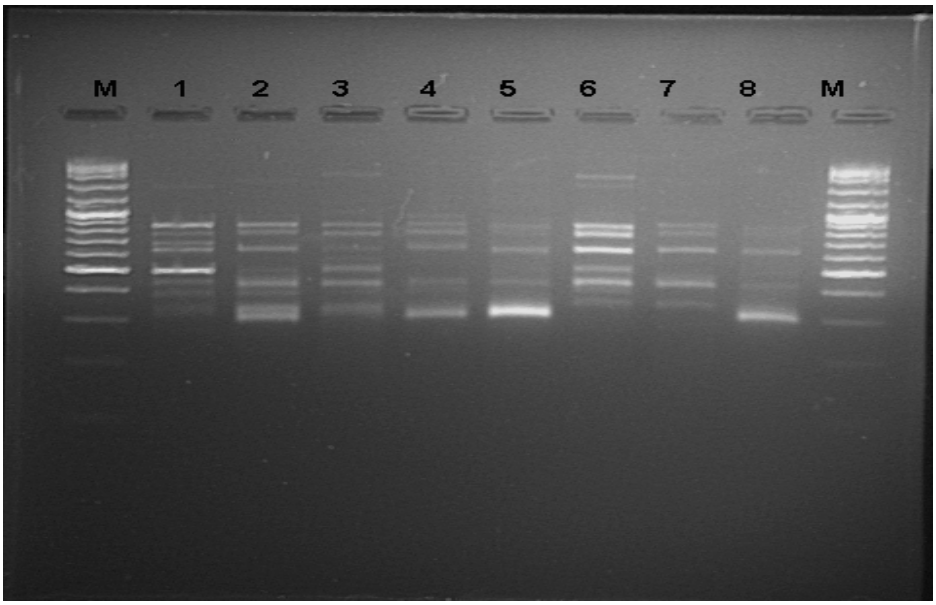

Figure 1. PCR amplification of random amplified microsatelite (RAM) of Asian green-lipped mussel using genomic DNA prepared from: lane 1 to lane 3: Chelex 100-Proteinase K-ethanol precipitation protocol from mussel A, B and C; lane 4 to lane 6: Chelex 100-Proteinase K-silica from mussel A, B and C; lane 7 to lane 8: Chelex 100-ethanol precipitation from frozen and ethanol-preserved mussel. Lane M is 100-bp DNA ladder. Mussel A, B, and C are three individual mussels preserved in ethanol

Quantitation of DNA

The quantity of DNA was determined by UV spectrophotometry (Thermo). One hundred microliters of DNA was added into tubes with 900 µl of sterile TE buffer to give a 1:10 dilution. Absorbance of the DNA dilution was measured at 260nm, 280nm and 320nm. The yield and purity of DNA were calculated as below:

DNA yield (µg) = $(A_{260} - A_{320})$ x 50 (DNA extinction coefficient) x dilution factor x final sample volume

DNA purity = A_{260}/A_{280}

Random amplified microsatelite (RAM)

A 5'-anchored oligonucleotide containing a dinucloetide repeat of 5'-NNN NNK KYW $(BD)_3$ $B(CA)_{10}$, in which K=(G or T), N=(A, C, G or T), Y=(T or C), B=(C, G or T), D=(A, G or T), was used for random amplified microsatellite analysis. The PCR reaction mixture consisted of 25 ng of genomic DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 µM primer, 100 µM of dNTP mix and 1.5 U of *Taq* DNA Polymerase (Promega), in a 10 µl final volume. PCR reactions were performed in a Veriti™ Thermal Cycler (Applied Biosystem). The amplification protocol was: 3 min at 94°C of pre-denaturation; 30 sec at 94°C, 60 sec at 65°C and 30 sec at 72°C for 35 cycles; and followed by a final extension of 5 min at 72°C. Amplification of PCR products were fractionated by electrophoresis on 2% of agarose gel and stained with ethidium bromide.

Results and discussion

In this study, different existing DNA extraction protocols had been compared and a rapid, simple and yet inexpensive protocol was developed by combining and modifying existing protocols. The developed protocol was tested on frozen and ethanol-preserved mussels and the results obtained on DNA purity and yield are summarised in Table 1. The protocol could extract genomic DNA with a higher purity from ethanol-preserved mussels (1.85±0.08) than from frozen mussels (1.71±0.08) However, both frozen and ethanol-preserved samples yielded comparable amounts of DNA (13.70±1.47 ng and 12.60±1.60 ng of DNA from frozen and ethanol-preserved mussels, respectively). Although the A_{260}/A_{280} ratio of DNA from frozen mussel was lesser than 1.8, the quality of the DNA extracted was still acceptable for PCR amplification. Amplification of random amplified microsatelite (RAM) by polymerase chain reaction

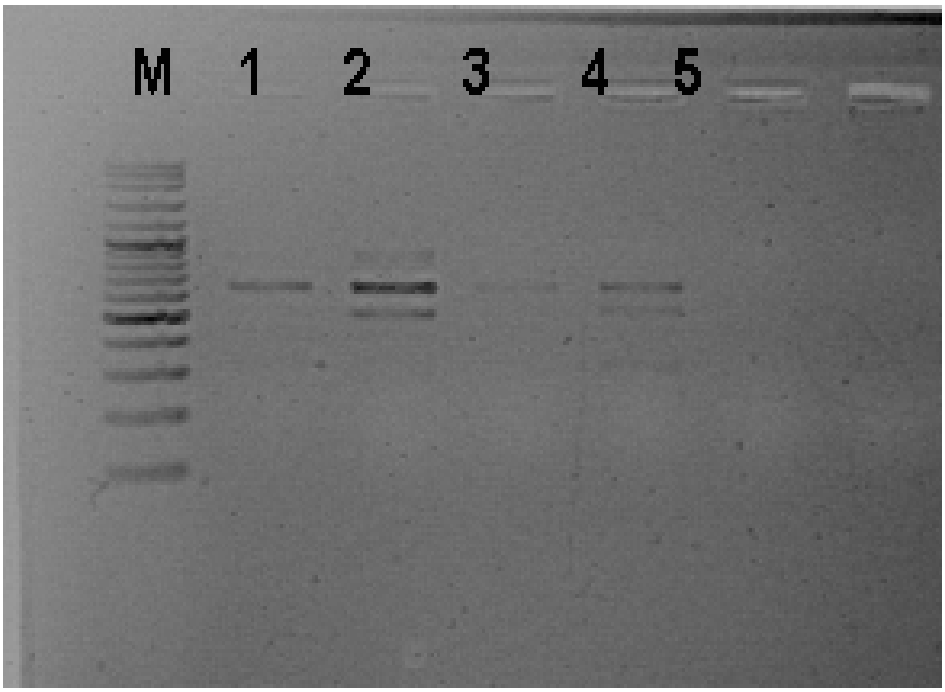

Figure 2. PCR amplification of random amplified microsatelite (RAM) of Asian green-lipped mussel using genomic DNA prepared from: lane 1 and lane 2: Chelex 100-Proteinase K protocol from frozen and ethanol-preserved mussel, respectively; lane 3 and lane 4: Proteinase K-ethanol precipitation protocol from frozen and ethanol-preserved mussel, respectively. Lane M is 100-bp DNA ladder

(PCR) yielded distinct bands on gel electrophoresis (Figure 1) and all the bands were reproducible.

The DNA extraction protocol was based on three steps: (1) boiling the mantle tissue in 6% Chelex 100 resin; (2) Tissue digestion with Proteinase K; and (3) DNA precipitation with absolute ethanol. The whole protocol took only approximately 1⅔ hours to complete and is suitable for DNA extraction from a large number of samples. Previous studies have reported successful PCR amplification using standard Chelex-100 resin method (Chelex 100 combined with Proteinase K digestion) for DNA isolation from ark shells and scallops (Steiner and Muller, 1996), larvae and juveniles of oysters (Launey and Hedgecock, 2001) and oyster parasites (Ko *et al*., 1999). In our experiments, however, DNA lysate obtained from Chelex 100-proteinase K digestion method yielded only a few weak bands in RAM analysis (Figure 2). Furthermore, the bands were not reproducible and yielded different profiles for each PCR amplification. UV spectrophotometry revealed low purity in the DNA extraction (1.22±0.09 and 1.37±0.05 from frozen and ethanol-preserved mussel, respectively) (Table 1). In fact, success in PCR amplification depends heavily on the quality of DNA template. Lower purity in DNA solution might inhibit PCR amplification. However, under certain circumstances, amplification by PCR can still take place by using powerful *Taq* DNA polymerase (Akalu and Ridchardt, 1999), optimization of PCR condition, increasing the concentration of poor-quality DNA (Fishback *et al*., 1999) and relief of PCR inhibitory effect by BSA and T4 gene 32 protein (Kreader, 1996). Poor-quality DNA might still be applicable for PCR detection of specific genes, but not suitable for molecular typing which requires high quality DNA to ensure reproducibility of clear multiple bands.

To explore the influence of Proteinase K and Chelex 100 resin to the entire protocol, we performed DNA extraction by omitting the use of Proteinase K or Chelex 100 resin. The purity of DNA obtained by both protocols was comparatively lower than the DNA obtained by Chelex 100-Proteinase K-ethanol precipitation protocol. Chelex 100 resin was proved to play a role in increasing the purity of prepared DNA. The protocol without usage of Chelex 100 resin yielded genomic DNA with A_{260}/A_{280} ratio of 1.69±0.20 and 1.61±0.09 from frozen and ethanol-preserved mussel. Proteinase K, on the other hand, was essential in protein digestion and releases more DNA from the tissue. When the boiling in Chelex 100 resin was followed by ethanol precipitation, the quantity of DNA extracted was significantly reduced

as compared to the full protocol (Table 1). The finding was in total agreement with the observation by Yue and Orban (2001).

The DNA extraction protocol combining Chelex 100 and proteinase K digestion, followed by silica purification was adapted and slightly modified from the protocol designed for DNA extraction from fish scales (Yue and Orban, 2001). The authors claimed that their method was rapid and yielded high quality DNA of approximately 50 to 700 ng per scale. In the present experiment, the method yielded only 8.67±1.45 ng and 9.40±0.56 ng of DNA from frozen and ethanol-preserved mussels, respectively. Furthermore, although higher purity was obtained by this method, it was comparatively more lengthy and expensive than Chelex 100-proteinase K-ethanol precipitation protocol. RAM profiles generated with DNA extracted by Yue and Orban's protocol were comparative with the profiles generated with our protocol. However, our findings were different from the outcomes reported by Aranishi and Okimoto (2006). The workers suggested that ethanol precipitation is unable to completely remove the PCR-inhibiting materials present in the mantle tissue that is rich in polysaccharide (Neudecker and Grimm, 2000) after failure in PCR amplification using the combined protocol of urea, Chelex 100 and proteinase K tissue digestion and followed by ethanol precipitation of DNA in the experiment. We speculated that the differences might be due to the use of urea and other chemical detergents in the digestion step that were not completely removed from the genomic DNA by ethanol precipitation and later inhibited the PCR amplification. In our protocol, the lysis step was merely performed by proteinase K in TE buffer and Chelex 100 resin. Thus, chemical contamination of final DNA product was not an issue in our procedure.

Our results demonstrate that the combined protocol of Chelex 100-Proteinase K-ethanol precipitation is a powerful yet economical method for DNA isolation from frozen and ethanol-preserved mussels for population phylogenetic studies. The procedure uses no hazardous chemical, as well as detergent in the DNA extraction and the full procedure takes not more than 2 hours to complete. Therefore, the protocol is suitable for investigating a large number of mussels for population genetic studies.

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