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Short Note

Efficient Methods for Isolating Mitochondrial DNA from Fresh or Fixed Molluscan Specimens

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Ling Jiang, Wen-Lung Wu and Yao-Sung Lin (1997) Efficient methods for isolating mitochondrial DNA from fresh or fixed molluscan specimens. *Zoological Studies* **36**(1): 74-78. Fast and efficient methods of isolating mtDNA from molluscan specimens with different conditions have been developed. The standard method involves isolation and purification of a large amount of mtDNA to a moderate purity from regular samples, even these containing jelly, mucus, or lipids; the 2nd method extracts mtDNA from a small quantity of sample, such as from 1 trochophore; and the 3rd method obtains mtDNA from formalin- or alcohol-fixed specimens.

Key words: Mollusks, mtDNA, Isolation.

The taxonomy and phylogeny of various classes and orders in Mollusca are still ambiguous due to incomplete fossil records, uncertain homology of morphological structures, or erasures of relationships over long periods of time (Burch et al. 1989, Boore and Brown 1994). The development of molecular biology methodology provides a new tool for approaching these problems (Moritz et al. 1987, Avise 1989, Harrison 1989). A reliable character that can reflect the evolutionary origin and the relationships among different molluscan taxonomic categories must be able to meet 4 conditions. The character must be: (1) present in all taxa; (2) absolutely homologous among all organisms; (3) complex enough to distinguish clear differences from convergences; and (4) changing with an investigated and known rate (Brown et al. 1979, Barton and Jones 1983, Boore and Brown 1994). Therefore, mitochondrial DNA (mtDNA) is likely to be an extremely useful molecule in providing high-resolution analyses for evolutionary relationships (Brown and Simpson 1981, Gyllensten et al. 1985, Moritz et al. 1987). Many mtDNA studies have concentrated on vertebrates. Only a little information on invertebrates is available, for which most is limited to economic aspects. There are only a few papers which have dealt with mtDNA in mollusks (Jiang et al. 1995). Hence, methods for isolating mtDNA from mollusks are needed for conducting studies in molluscan systematics. The 3 methods presented below can isolate mtDNA from molluscan organisms under different conditions, such as specimens with a very small quantity of tissue; specimens containing jelly, lipids, or mucus; specimens with very hard foot muscles; or specimens fixed in either alcohol or formalin.

Materials and Methods—For convenience, Taiwan abalone, *Haliotis diversicolor* Reeve, 1846, the main experimental animal in our laboratory, was used.

Standard method

Standard method was modified from Chapman and Powers (1984) and Jiang (1990). This general protocol is suitable for most specimens, as well as molluscan specimens containing jelly, lipids, or mucus; and the amount and purity of mtDNA isolated is adequate for restriction enzyme digestion. **Homogenization:** Although liver is a suitable organ for isolating mtDNA from many animals, the digestive gland of mollusks is not. The molluscan digestive gland contains many kinds of digestive enzymes that will degrade DNA, and the molluscan stomach may contain other organisms which have DNA that may be amplified in the PCR process. If specimens contain mucus, it should be removed by washing with Prep buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.5% Tween 20). Instead of liver, abalone ovaries (0.5 g) were homogenized with 10 ml of ice-cold MSB-EDTA buffer (210 mM mannitol, 70 mM sucrose, 50 mM Tris-HCl pH 7.5, 100 mM EDTA) in a 15-ml glass Teflon homogenizer. The homogenate was checked with a compound microscope to make sure that all cell membranes were broken to release cell organelles. **Mitochondria isolation:** The homogenized solution was transferred to a 15-ml sterile conical centrifuge tube, and 15% sucrose-MSB-EDTA buffer was added from the bottom of the homogenate to a total volume of 15 ml. This was then centrifuged at 1 000 x g at 4 °C for 10 min. After centrifugation, the solution could be seen to be separated

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into 5 layers: a light-yellow lipid layer, a dark mitochondrial layer, a sticky chromosomal layer, a clear sucrose-MSB-EDTA layer, and a debris pellet. The mitochondrial layer was transferred to a 50-ml centrifuge tube. TEK buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 1.5% KCl) was added to a total volume of 35 ml. This was then centrifuged at 15 000 x g at 4 °C for 1 h. Since oocytes contain large quantities of glycogen, a loose mitochondrial pellet would settle on top of a dense clear pellet. The loose mitochondrial pellet was drawn off and resuspended in TEK buffer to 35 ml and centrifuged at 15 000 x g at 4 °C for 30 min. **Mitochondrial DNA purification:** The mitochondrial pellet was transferred to a 2.0-ml Eppendorf tube, resuspended in TEK buffer (1.7 ml), and 1/20 volume of 18% SDS, and proteinase K were added to a final concentration of 100 μg/ml. The suspension was incubated at 55 °C for 1 h and then centrifuged with an Eppendorf centrifuge at 10 000 x g at room temperature for 10 min. The supernatant was transferred to a new Eppendorf tube, an equal volume of Tris-phenol/isoamyl alcohol/chloroform (50:1:24) was added, and this was allowed to mix well for 5 min. This solution was then centrifuged with an Eppendorf centrifuge at 10 000 x g for 10 min. The supernatant was transferred to a new Eppendorf tube and an equal volume of isoamyl alcohol/chloroform (1:24) was added and the mixing and centrifugation processes were repeated (10 000 x g for 10 min). Finally, the supernatant was transferred to a new Eppendorf tube, and an equal volume of isopropanol was added, mixed, and let stand for at least 10 min. Then, this was centrifuged at 10 000 x g at 4 °C for 20 min. After centrifugation, the supernatant was discarded. The mtDNA pellet was washed with 70% ethanol, vacuum dried, then resuspended in 50-100 μl TE buffer.

Method 2 for speciman of small quantity

Molluscan mtDNA can be isolated from a small quantity of tissue, even as small as 1 trochophore. A small portion of an epipodium was cut from the mantle. The tissue was washed with distilled water, then with MSB-EDTA homogenization buffer. The specimen was homogenized with 50 μl MSB-EDTA buffer in a 1.5-ml Eppendorf tube on ice. Proteinase K to 50 μg/ml, 1/5 total volume of 10% SDS, and 1/5 volume of 5 M NaCl were added to the homogenized solution, then incubated at 56 °C for 1 h. It is important to make sure that the solution becomes transparent. The homogenate was centrifuged at 10 000 x g for 10 min. The supernatant was transferred to a new Eppendorf tube, phenol/chloroform extraction, mtDNA precipitation, drying, and resuspension procedures were carried out as those mentioned above, except 2 volumes of cold (−20 °C) ethanol were added instead of isopropanol and then the mixture was allowed to sit at −70 °C for at least 30 min.

Method 3 for specimens preserved in alcohol or formalin

Method 3 was modified from Heller et al. (1991). With this method we succeeded in isolating mtDNA from more than 10-year-old, formalin-preserved specimens. A piece of muscle (40 mg) was cut from the foot of a Taiwan abalone. The specimen was washed with distilled water, then with Prep buffer. It was homogenized (20-50 mg tissue/0.5 ml Prep buffer with 0.5 mg/ml Proteinase K) in a 1.5-ml Eppendorf tube. The homogenized solution was sonicated for 10 min at 45 °C, then boiled for 10 min. It was then centrifuged for 20 min at room temperature, and the supernatant removed to a new Eppendorf tube. SDS (18%) solution was added dropwise to the supernatant at 56 °C until it became transparent. Then, the remaining phenol/chloroform extraction, alcohol precipitation, drying, and resuspension procedures were performed as describe above. The mtDNA purified with the last 2 methods was pure enough to use for PCR.

Primers and kits for polymerase chain reaction (PCR), PCR product elution, and PCR-directed sequencing

The pair of primers consisted of 16S L21 (5'-TAATGGTC GAACAGACCAACC-3') and 16S R21 (5'-CGCCTGTTTATCAAA AACAT-3'). This pair of primers are suitable for amplifying part of the 16S rRNA (400-600 bp) in all Archaeogastropoda and yielded good sequencing results. Amplification was performed with Super Taq polymerase and 10X reaction buffer, both of which were supplied by HT Biotechnology Ltd., USA. A Geneclean III commercial kit (Bio 101, Vista, CA, USA) was used for the elution step. PCR-directed sequencing was performed using a Perkin Elmer AmpliCycle sequencing kit (Roche Molecular Systems, Inc., Branchburg, NJ, USA).

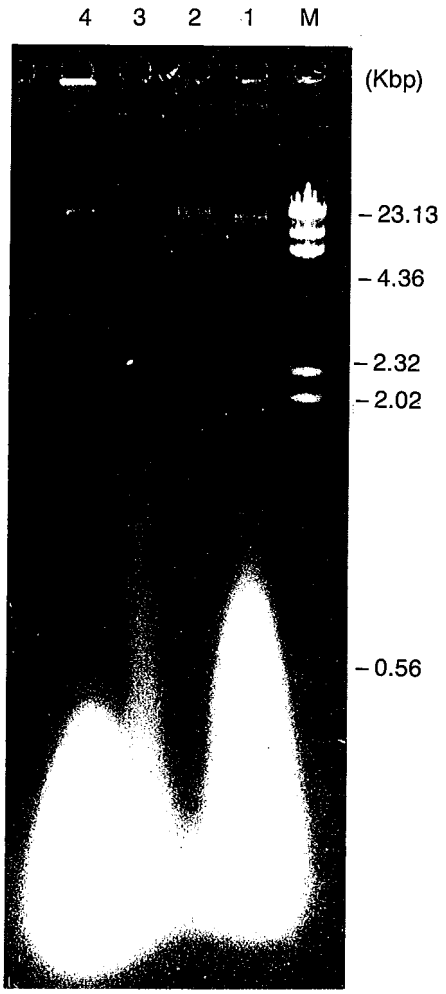

Fig. 1. Photograph of ethidium bromide-stained gel of mtDNA.
M: λ/*Hind* III molecular marker;
Lane 1: mtDNA isolated by the standard method;
Lane 2: mtDNA by method 2 from small-quantity specimens;
Lane 3: mtDNA by method 3 from formalin-preserved specimens;
Lane 4: mtDNA by method 3 from fresh specimens.

Results—After mtDNA isolation, 2 μl from a total volume of 200 μl mtDNA-TE solution from the standard method (lane 1), 5 μl from a total volume of 50 μl mtDNA-TE solution from method 2 (lane 2), 5 μl from a total volume of 50 μl mtDNA-TE solution from method 3 (lane 3), and 10 μl from a total volume of 400 μl mtDNA-Prep-buffer solution from method 3 (lane 4) were electrophoresed in a 0.8% agarose gel in 1x TBE buffer (Fig. 1). MtDNA of lane 4 was isolated from a fresh abalone sample (about 40 mg foot muscle) using the 3rd method except that the SDS lysis step, phenol/chloroform extraction, and ethanol precipitation were not carried out. This modification of the 3rd method is suitable only for obtaining crude and small amounts of mtDNA from fresh or frozen specimens for PCR. MtDNA of lane 3 from formalin-preserved abalone could hardly be seen and DNA degradation was severe. The quantities of mtDNAs of lanes 2, 3, and 4 were suitable only for PCR and PCR sequencing.

The large amount of RNA at the bottom of the gel can obscure small molecular-weight fragments from RFLP, and it can be eliminated by RNase A treatment (Figs. 1, 2) (Sambrook et al. 1989). Figure 2 presents a successful isolation with the standard method, and all the restriction fragments can be seen clearly.

Solutions of 2, 10, 10, and 10 μl mtDNA corresponding to lanes 1, 2, 3, and 4 respectively, of Figure 1 were used as templates in the PCRs. Products from PCRs were analyzed and are shown in Fig. 3. DNA bands of 500 bp of various intensities can be observed in all lanes. Also, PCR sequencing using 4-template samples was carried out; the results were analyzed and are shown in Figure 4.

Discussion and Conclusion—Oocytes of mollusks usually contain large amounts of lipids which can be removed by a 15% sucrose centrifugation. Lipids will form a viscous layer at the surface of the centrifuged solution which can be easily removed with a spatula. If specimens contain a large quantity of glycoproteins and polysaccharides, phenol/chloroform extraction should be repeated several times until the white protein layer between the mtDNA solution and the phenol/chloroform layer disappears. Mucus in specimens can be removed before homogenization by washing with Prep buffer several times until all the mucus disappears.

The standard method is suitable for all samples which contain a large quantity of tissue. Using this method, we have

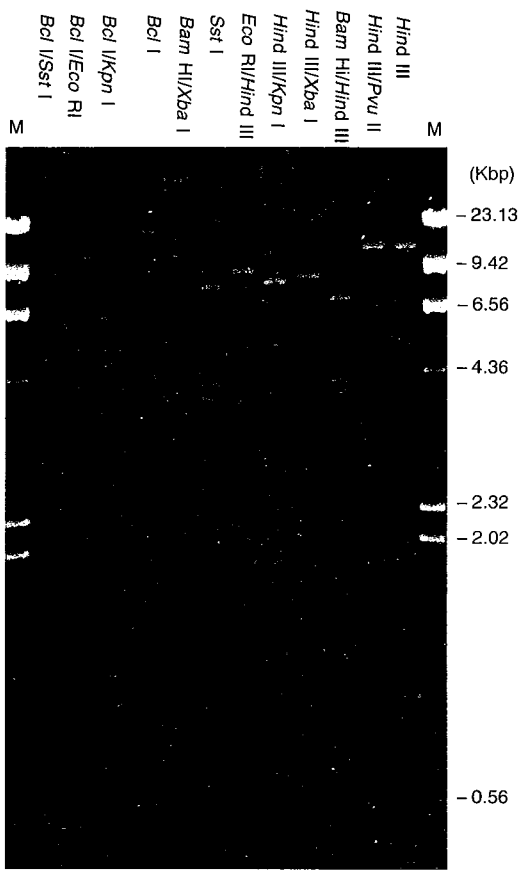

Fig. 2. Photograph of ethidium bromide-stained gel of mtDNA from land 1, Figure 1 digested by *Hind* III, *Pvu* II, *Bam*H I, *Xba* I, *Kpn* I, *Eco*R I, *Sst* I, and *Bcl* I with single or double enzymes. M: λ/*Hind* III molecular marker.

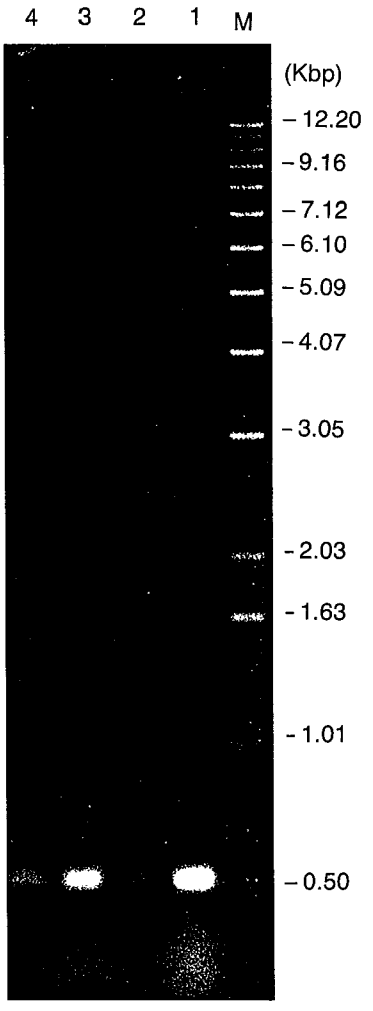

Fig. 3. Photograph of ethidium bromide-stained gel of PCR products from mtDNAs in Figure 1.
M: 1-Kb ladder molecular marker.

successfully isolated mtDNAs from different animals, including vertebrates (frogs, ducks, and carp) and invertebrates (mollusks, fruit flies, earthworms, and sea urchins). MtDNA purified with this method can be digested with restriction enzymes. If only a small amount of a specimen is available, the 2nd method is recommended. We have successfully isolated mtDNAs from a fresh water limpet *Ferrissia* (smaller than 1 mm in shell length) and an oyster trochophore with the 2nd method. The 3rd method for specimens preserved in alcohol or formalin is suitable for either fresh or frozen specimens which contain mucus. We have isolated mtDNAs from fresh specimens of cowries (Cypraeidae), sea slugs (Aplysiidae), and terrestrial slugs (Philomycidae) with this method. However, the quantity of mtDNA produced with the 2nd and 3rd methods is adequate only for PCR and PCR sequencing.

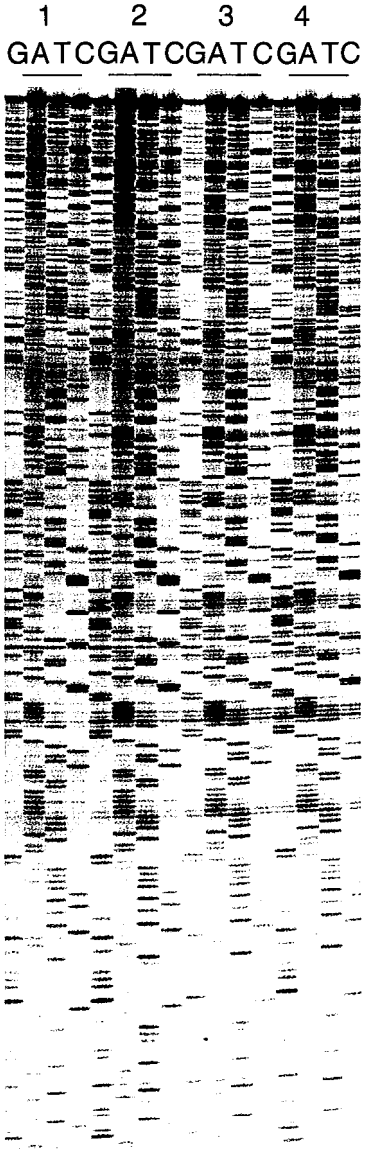

Fig. 4. Photograph of sequencing gel from PCR products in Figure 3.

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從不同性質之軟體動物標本抽取粒線體 DNA 之方法

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本文針對性質不同的動物標本，提供三種快速而有效的 mtDNA 抽取方法，期望經由這些技術的建立，使得 mtDNA 相關的研究工作，被更加廣泛地運用。第一個方法，適用於一般較大量的動物標本，即使含有膠質、黏液、或脂肪，亦可得到大量、純淨的 mtDNA。第二個方法，能夠從像一個擔輪子幼蟲那麼少量的標本中抽取出 mtDNA。第三個方法，可從長期被酒精、或福馬林固定的標本中，僅需三個小時就可抽取出 mtDNA。

關鍵詞：軟體動物, mtDNA, 抽取方法。

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