DNA Extraction from Different Preserved Tissue of *Cassidula aurisfelis* for PCR study

Wan Bayani W. Omar[1], Mohammad Shafie B. Shafie[2,*] and Zaleha Kasim[3]

[1]Department of Biological Sciences, Faculty of Science and Technology,
[2]Department of Agrotechnology, Faculty of Agrotechnology and Food Science,
[3]Institute of Aquaculture, Universiti Malaysia Terengganu,
21030 Mengabang Telipot, Kuala Terengganu, Malaysia
*msshafie@yahoo.com.my

Abstract: Preserving DNA within tissue make the ability to collect and stabilize samples in the field or operating room, also making it easier to use the sample for histology and DNA isolation. Two preservative methods, Ethanol 95% and TNES-Urea Buffer were used. The best method makes DNA quality clear and sharp on gel electrophoresis. TNES-Urea buffer make clear pattern. Two extraction methods were using for yield, quality and suitability of genomic DNA for RAPD marker amplification in *Cassidula auresfellis.* Phenol chloroform method makes clear and sharp DNA quality for preserved samples (Ethanol 95% and TNES-Urea Buffer) and the purity between 1.0847 - 1.6715. Screening of RAPD marker produced 90% in TNES-Urea Buffer samples and 70% in Ethanol 95%.
[Nature and Science. 2009;7(9):8-14]. (ISSN: 1545-0740).

Key words: Preservation, DNA extraction, PCR, RAPD, snail.

1. Introduction

Cassidula aurisfelis is known as Angulated Shoulder Ear Snail, Angulate Vassidula or Cat's Ear Cassidula in English (Smith, 1992). It belongs to the great division or phylum called the Mollusca and from class of gastropoda. The shell is one piece (univalve) and may be coiled and uncoiled. Body bilaterally symmetrical and unsegmented, usually with definite head. Feeding habits of snails are as varied as their shape and habitats, but all include the use of some adaptation of the radula.

Owing to recent innovation in molecular biological techniques, such as polymerase chain reaction (PCR) and DNA automated sequencing, nucleic acid data are becoming more and more important in biology (Hillis *et al.,* 1996). One of the modern marker techniques for studying genetic variability is Random Amplified Polymorphic DNA, RAPD (Williams *et al.,* 1990). The technique requires no prior knowledge of the genome and it needs only a small amount of DNA (Hadrys *et al.,* 1992). Using this technique polymorphism can be detected in closely related organism.

Preservation is really important procedure to make samples keep on original quality. According to Dessaure *et al*., (1996), preservation of tissues for DNA extraction is important because it can protect these potentially valuable resources. In this study, there are two preservative were used to examine and determined the effects to DNA of the samples (TNES-Urea buffer and ethanol 95% solution). Usually TNES-Urea buffer were used in fish preservation. These study were detect the effected of TNES-Urea buffer to the DNA of mollusk. Ethanol solutions are one of the methods for tissue preservation for DNA analysis. Ethanol is suitable to the storage of vertebrate tissue and has been used successfully in DNA hybridisation and sequencing (Dessaur *et al*., 1996). 95 - 100% ethanol at ambient temperature were used to tissue samples from invertebrate for molecular studies (Winsor, 1998).

2. Materials and Methods

2.1 Sample Collections.

The samples of *Cassidula aurisfelis* were collected randomly from the area in Setiu Wetland, Setiu, Terengganu. 15 individuals were collected randomly around this area by hand packing. All the samples were collected during the low tide of water. The length, width, thickness and weight from each sample were measured.

2.2 Preservation

There are two preservative for preservation, Ethanol 95% and TNES-Urea Buffer. Ethanol 95% were prepared by dilutes 100% Ethanol to 95% Ethanol. The solution of TNES-Urea Buffer (Tris ; for 200 ml : 2 ml of 1 M pH 7.5 ; final conc. : 10 mM, NaCl ; for 200 ml : 5 ml of 5 M ; final conc. : 125 mM, EDTA-2Na ; for 200 ml : 2 ml of 0.5 M pH 7.5 ; final conc. : 10 mM,

SDS ; for 200 ml : 10 ml of 10 % ; final conc. : 0.5 %, Urea ; for 200 ml : 48.05 g ; final conc. : 4 M) were mixed (Asahida *et al*., 1996). This samples preservation was saved for three and four month.

2.3 Kit Wizard™ Genomic DNA Purification (Promega)

DNA from all snail body tissue was extracted from the samples by using Kit Wizard™ Genomic DNA Purification (Promega). About 70 mg of all body tissue were used for the extraction of the DNA. 600 µl of nuclei lysis were added to the all body tissue into 1.5 ml micro centrifuge tube. The mixtures then were homogenized to get the lysat. Then the sample was incubated in the water bath at 65°C for about 15 to 20 minutes. After that, it was treated with 3.0 µl of RNase. The sample then was incubated again in water bath at 37°C for 15 to 20 minutes. Next is the sample was left at room temperature for 5 minutes.

About 200 µl Protein Precipitation were added in the sample and then the sample were vortex at highest maximum speed for about 20 seconds. Then the sample was centrifuged at 14,000 rpm at room temperature for 3 minutes. The supernatant that contain DNA will be removed to put into a new micro centrifuge which contains 600 µl of isopropanol. The sample was centrifuged once again at 14,000 rpm at room temperature for 2 minutes. Next step is 600 µl of ethanol (70 %) were added to the pellet to wash the DNA. Once again the sample was centrifuged at 14,000 rpm at room temperature for 1 minute. Then the DNA was dried at room temperature for 10 to 15 minutes. Then the DNA was resuspended with 100 µl of DNA rehydration for 1 hour. The DNA extraction sample was keep at -20°C to avoid DNA from degradation.

2.4 Phenol-Chloroform Method

DNA was extracted based on the Phenol-chloroform method described by Brown *et al.* (1991) with some modifications. Digestion buffer at volume of 500 µl containing (1 % (w/v) Sodium Dodecyl Sulphate 0.8 %, Triton X-100, 0.5 M NaCl, 0.1 M Tris-Hcl at pH 9, 0.01 M EDTA) were added into 1.5 ml microcentrifuge tube which containing 70 mg all snail body tissue and then the 40 µl of 10 % (w/v) SDS and Proteinase K (20 mg/ml solution) were added. The tube was shaken gently and was incubated at 55^0C for 1 to 2 hours. The sample was treated with 25 µl of RNase. Then, the mixture was left at room temperature for 15 to 30 minutes. The sample were treated with 500µl of phenol:chloroform:isoamyl alcohol (25:24:1) and gently the tube were vortexed to homogenize.

The sample was left at room temperature for 10 minutes before doing centrifugation at 13,000 rpm for 5 minutes. The top later is aqueous and were remove and dispersed into the new microcentrifuge tube. The step of adding phenol:chloroform:isoamyl alcohol were repeated twice. The samples were treated with 500 µl of chloroform:isoamyl alcohol (24:1) and were centrifuged at 13,000 rpm for 5 minutes. The upper aqueous layer was mixed with 1 ml of ice-cold absolute ethanol by rapid inversion of the tubes several times. Then, centrifuge at 6,000 rpm for 30 minutes and after that the precipitated DNA were collected at the bottom tubes as a white pellet. The pellet was washed with 500 µl of 70 % of ethanol and was centrifuge at 6,000 rpm for 15 minutes. The DNA was allowed to dry at room temperature. Then resuspended with 100 µl TE buffer (10 mM Tris and 1 mM EDTA, pH 8) for at least 24 hours at room temperature to fully dissolved before proceeding to the next step. This DNA extraction samples will be kept in – 20^0C to avoid DNA degradation.

2.5 Measurement of DNA Purity and Quality

The samples were separated by agarose gel electrophoresis through 1.0 % of agarose gel in 1.0 X TBE. Electrophoresis was run at 55 volts for 1 to 2 hours. Then, the gel was stained with ehidium bromide for 20 to 30 minutes and washed with distilled water for 5 to 10 minutes. The gel was photographing with Image Master VDS (American Pharmacia, Biotech).

The genomic DNA was quantified using UV-spectrophotometer. The quantity of DNA were measured by obtaining the absorbance reading at 260 nm and the purity of DNA were estimated by calculating the ratio of absorbance reading at 260nm and 280nm. The quantification can be determined base on ratio ($OD_{260}/_{280}$). An OD of 1 corresponds to approximately 50 µg/ml for double-stranded DNA (Sambrook *et al.*, 1989). The DNA concentration was determined by the formula:

DNA concentration = OD_{260} × 50 µg/ml × dilution factor (Linacero *et al.*, 1998).

2.6 Screening of RAPD Primers

10 RAPD primers (Table 1) from Operon Technology were screened from a single individual. Primers that have the basic of sharpness, clarity of the profile and the existence of polymorphism usually were chosen for further study. (D'Amato and Corach, 1997).

The total reaction volume of 25 µl were used with the final concentration containing 1.0 × of reaction buffer included the concentration of genomic DNA 50 ng, Fermentas Magnesium Chloride 4.0 mM, Fermentas Taq DNA Polymerase 2 units, Fermentas dNTP-mixture 0.4 mM and primer 10 pM.

The DNA was amplified by using a Master Cycles

Gradient (Eppendorf). The amplification were programmed at 45 cycles for 30 seconds of denaturation at 94°C, 30 seconds of annealing temperature at 36°C, 1 minutes of primers extension at 72°C and final extension of 2 minutes at 72°C.

Table 1 Code, sequence, nucleotide length and G+C content of primers used in RAPD analysis

No.	Primer Code	P Primer sequence 5' to 3'	Nucleotide length	G+C content (%)
1	OPA 01	CAGGCCCTTC	10-mers	70
2	OPA 02	TGCCGAGCTG	10-mers	70
3	OPA 03	AGTCAGCCAC	10-mers	60
4	OPA 04	AATCGGGCTG	10-mers	60
5	OPA 05	AGGGGTCTTG	10-mers	60
6	OPA 06	GGTCCCTGAC	10-mers	70
7	OPA 07	GAAACGGGTG	10-mers	60
8	OPA 08	GTGACGTAGG	10-mers	60
9	OPA 09	GGGTAACGCC	10-mers	70
10	OPA 10	GTGATCGCAG	10-mers	60

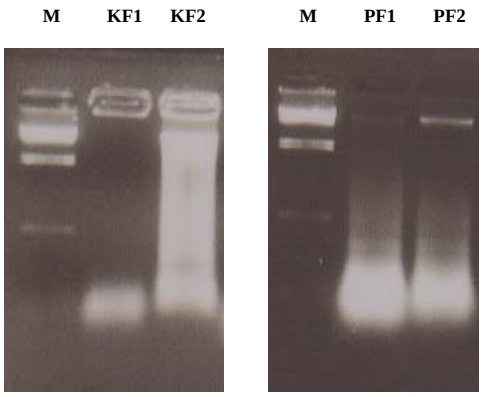

Figure 1 Genomic DNA extracted for fresh tissues, λ DNA/Hind III marker (lane M), (K - Wizard Genomic DNA Purification Kit (Promega) protocol, P – Phenol Chloroform Method, F – Fresh tissue).

3. Result and Discussion

3.1Extraction of DNA

The DNA extraction method was obtained from Kit Wizard™ Genomic DNA Purification and Phenol-chloroform Method. The Genomic DNA was successfully extracted and observed to have impurity and purity. Using Phenol-chloroform method, the clear band and high purity of DNA was obtained. According to Zhang and Hewitt (1998), the samples collected from remote areas have to be preserved before DNA analysis is carried out. The fresh tissue, muscles or blood sample provides the best source of DNA biological analysis (Parenrengi, 2001).

Fresh samples extraction had shown (Figure 1) no DNA and degraded band for Kit Wizard™ Genomic DNA Purification. For Phenol-chloroform method, the samples also degraded. The degraded for fresh samples usually causes by contamination of fresh samples by contain other particles when doing extraction.

Extraction using Kit Wizard™ Genomic DNA Purification for preserved sample in TNES-Urea Buffer produced degraded band for third month preserved and no DNA for fourth month preserved on the electrophoresis gel. However, sample preserved in 95% ethanol show no DNA and degrade band for third and fourth month preserved (Figure 2).

Phenol-chloroform had a good result, the DNA clear banding pattern was obtained in third and fourth month in TNES-Urea buffer and Ethanol 95% preserved samples. Clear banding pattern are shown in Figure 3.

Samples preserved in TNES-Urea buffer yielded proper and nice band fragments on the electrophoresis agarose gel. TNES-Urea buffer consist a few chemicals such as Tris-HCl, Natrium Chloride, EDTA, Sodium Dedocyl Sulphate, which had an agent that assimilate the whole tissue samples and DNA. All the samples that been preserved in TNES-Urea buffer were fully digested in the solution. These make easy to lyses the muscle of samples.

For samples preserved in ethanol 95% were producing improper result due to degradation. All the tissue samples in ethanol 95% became hard and maintain condition as long as it had been preserved but the ethanol solution sometimes is evaporated and dried. Tissue samples in ethanol not easy to be lyses and homogenized.

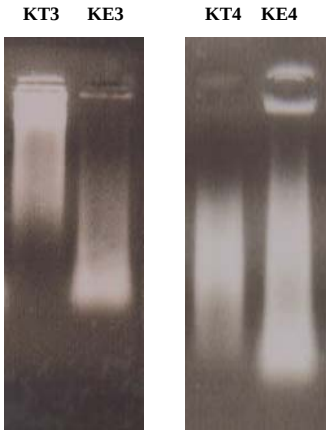

Figure 2 Genomic DNA extracted for Kit Wizard™ Genomic DNA Purification, λ DNA/Hind III marker (lane M), (T – TNES-Urea Buffer, E – Ethanol 95%, 3 – Third month, 4 – Fourth month).

During organic extraction, protein contaminants are denatured and partition either with the organic phase or at the interface between organic and aqueous phases, while nucleic acids remain in the aqueous phase. Phenol used in this protocol is buffered to prevent oxidized products in the phenol from damaging the nucleic acids (University of Regina, 1998).

3.2 Purity and Quantity of DNA

The DNA purity using Wizard Genomic DNA Purification Kit (Promega) was ranged from 1.1870 to 1.3554, while DNA purity with Phenol-chloroform Method was from 1.0847 to 1.6715. The range was estimated quantitatively from the ratio between the reading of absorbancy at 260nm and 280nm ($OD_{260}/_{280}$) in UV-Biophotometer. Quantity of DNA with Wizard Genomic DNA Purification Kit (Promega) calculated ranged from 337.50 to 906.00µg/mL while quantity of DNA with Phenol-chloroform Method ranged from 160.00 to 1005µg/mL. Through the result obtained, *Cassidula aurisfelis* contains high concentration of

DNA using Phenol Chloroform Method. This high concentration of DNA will banding pattern of DNA amplification. The values of the DNA purity and quantity of Kit Wizard™ Genomic DNA Purification and Phenol-chloroform Method are shown in Table 2.

Previous studies suggested the use of genomic DNA ranging from 1.8 to 2.0 in purity for PCR requirement in amplification of DNA. Purity of genomic DNA lower than 1.8 was contaminated with protein, while more than 2.0 purity of genomic DNA was suspected to be contaminated with organic matters residue, derived from the DNA extraction (Sambrook *et al.*, 1989).

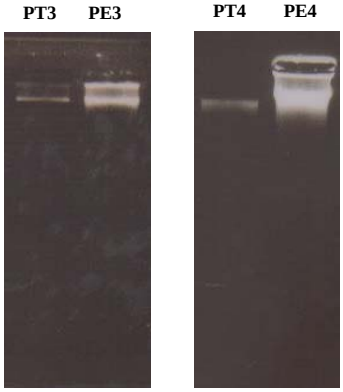

Figure 3 Genomic DNA extracted for Phenol-Chloroform method, λ DNA/Hind III marker (lane M), (T – TNES-Urea Buffer, E – Ethanol 95%, 3 – Third month, 4 – Fourth month).

In this case, the causes for obtain high quality DNA such as food or feces remaining inside the abdomen, which could promote the degradation of the DNA and contribute to contamination (Zhang and Hewitt, 1998). For the poor quality, according to Pearson and Sterling (2003), some tissues contain large amount of connective tissue and are difficult to digest, these can be ground in a mortar and pestle before being digest with nuclei lysis.

3.3 Screening of RAPD Primers

Ten primers from the Operon 10 mers (Operon Kit A) (OPA 01 to OPA 10) with 60% – 70% GC content were used during the screening of the RAPD primers. Screening using TNES-Urea Buffer produced more amplication band on gel electrophoresis (Figure 4). The site of band shown between 150 – 1200bp. The degrade DNA from preserved samples in Ethanol 95% (Figure 5), shown poor result which only OPA 02, OPA 03, OPA 04, OPA 07, OPA 08, OPA 09 and OPA 10 produced band on gel electrophoresis. The site of band shown between 250 – 1200bp.

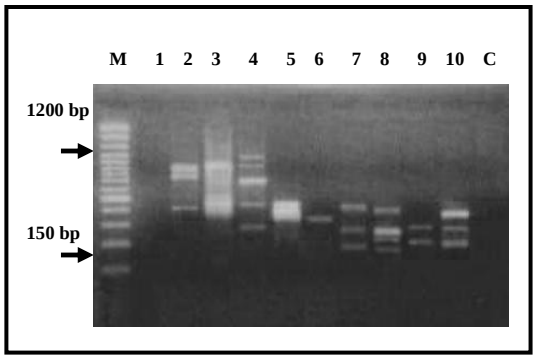

Figure 4 RAPD (TNES-Urea buffer) banding patterns for screening of 1st base primers, OPA 01 to OPA 10 (lane 1 to 10). (Lane M is a marker 100bp ladder plus, C - Control).

Lower number of amplicons in extracted DNA of preserved tissue in TNES-Urea buffer and Ethanol 95% suggests presence of contaminants like polysaccharide and polyphenols as well as RNA, which inhibits *Taq* polymerase (Scott and Playford, 1996). DNA quality is a major factor in genetic analysis using molecular markers in earlier reports on plants (Weeden et al., 1992; Staub et al., 1996) and it same with animals.

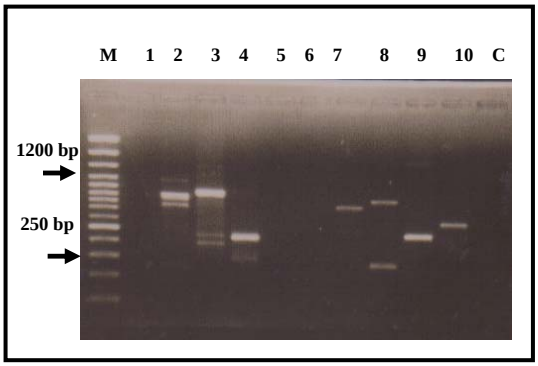

Figure 5 RAPD (Ethanol 95%) banding patterns for screening of 1st base primers, OPA 01 to OPA 10 (lane 1 to 10). (Lane M is a marker 100bp ladder plus, C - Control).

Some polymorphisms were easy to score whereas other bands appeared to produce ambiguous fragments (William *et al*., 1990). The best primers will produce more than three fragments and clear. The number of fragments generated is dependent on the primer sequence rather than to the nucleotide length.

Table 2 Observed density (OD) of purity and quantity of DNA for Genomic DNA extracted by Kit Wizard™ Genomic DNA Purification and Phenol-chloroform Method.

Sample	Average OD_{260}	Average OD_{280}	Ratio OD_{260}/OD_{280}	Quantity DNA(µg/mL)
KF1	0.1365	0.1090	1.2523	341.25
KF2	0.1880	0.1490	1.2617	470.00
KT3	0.1350	0.1030	1.3107	337.50
KE3	0.3205	0.2700	1.1870	801.25
KT4	0.1640	0.1210	1.3554	410.00
KE4	0.3600	0.2820	1.2766	906.00
PF1	0.1970	0.1390	1.4173	492.50
PF2	0.1230	0.0950	1.2947	307.50
PT3	0.0640	0.0590	1.0847	160.00
PE3	0.0935	0.0820	1.1402	233.75
PT4	0.1270	0.0970	1.3093	317.50
PE4	0.4020	0.2405	1.6715	1005.00

Table 3 Number of bands for *Cassidula aurisfelis* generated from OPA 01 - OPA 10 in differences preservation extracted using Phenol-Chloroform Method.

Number of band	OPA 1	OPA 2	OPA 3	OPA 4	OPA 5	OPA 6	OPA 7	OPA 8	OPA 9	OPA 10
TNES	0	3	2	4	2	1	3	3	2	3
Ethanol 95%	0	3	3	2	0	0	1	2	2	1

Correspondence to:
Mohammad Shafie B. Shafie
Department of Agrotechnology,
Faculty of Agrotechnology and Food Science,
Universiti Malaysia Terengganu,
21030 Mengabang Telipot,
Kuala Terengganu, Malaysia
Emails: msshafie@yahoo.com.my

References

1. Asahida, T., Kobayashi, T., Saitoh, K., and Nakayarma, I. (1996). Tissue preservation and total DNA extraction from fish stored at ambient temperature using buffers containing of urea. Fisheries Sci 62: 727-730.
2. Brown, T. A (1991). Essential Molecular Biology, A Practical Approach. *Oxford University Press*. Ney York, pp. 71.
3. D'amato, M.E ; Corach, D. (1997) Population genetic

structure in the fresh water *anomuran aesla jujuyana* by RAPD analysis.

4. Dessauer HC, Cole CJ, Hafner MS (1996) Collection and storage of tissues. In: (eds Hillis DM, Moritz C, Mable BK) *Molecular Systematics*, 2nd edn, pp. 29–47. Sinauer Associates, Sunderland.
5. Greer CE, Lund JK and Manos MM (1991) PCR amplification from paraffin embedded tissues: recommendations on fixatives for long-term storage and prospective studies. *PCR Methods* and Application.
6. Hadrys, H., Balick, M. and Shierwater, B. (1992). Aplications of Random Amplified Polymorphic DNA (RAPD) in Molecular Ecology 1:55-63.
7. Hillis DM, Moritz C, Mable BK (1996) *Molecular Systematics*, 2nd edn. Sinauer Associates, Sunderland.
8. Linacero, R. J. Rueda and A. M. Vazquez. (1998). Quantifying of DNA. Karp, A., P. G. Isaac, and D. S Ingram (Eds.) Molecular Tools for Screening Biodiversity: Plants And Animals. Chapman and Hall. London, Weinheim, New York, Tokyo, Melbourne, Madras. pp 18-21
9. Parenrengi A. (2001). Study on Genetic Varibility of Groupers (*Epinephelis* spp.) From Malaysia And Indonesian Waters Using PCR-RAPD Analysis. Faculty Science and Technology, University Putra Malaysia.
10. Sambrook, J., E.F. Frilsch, and T. Maniatis. (1989). Molecular Cloning: A Laboratory Manual, 2nd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
11. Scott KD, Playford J (1996). DNA extraction technique for PCR in rain forest plant species. Biotechniques, 20: 974-978.
12. Smith, B.J., (1992). Non-marine Mollusca. In: Houston, W.W.K., Editor. *Zoological Catalogue of Australia*, Vol. 8: 1-398. Australian Government Printing Service, Canberra. 405pp. Now Vol. 17.1.
13. Staub JJ, Bacher J, Poeter K (1996). Sources of potential errors in the application of random amplified polymorphic DNAs in cucumber. Hort.Sci. 31: 262 – 266.
14. University of Regina (October 30, 1998). Phenol/Chloroform Extraction. and Ethanol Precipitation. Description. Phenol/Chloroform Extraction. [online]. University of Regi. http://uregina.ca/~ngdann/Bioc422/proj1.htm [Accessed on 25th November 2005].
15. Weeden NF, Timmerman M, Hermmat M, Kneen BE, Lodhi MA (1992). Applications of RAPD Technology to Plant Breeding, Symposium Proceeding, Minneapolis. pp. 12 –17.
16. Williams, J.G.K., A.R. Kubelik, K.J Livak, J.A. Rafalski, and S.V. Tingey. (1990). DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Research*. 18:6531-6535.
17. Winsor L (1998) collection, handling, fixation, histological and storage procedures for taxonomic studies of terrestrial flatworms (Tricladida, Terricola). *Pedobiologia.*
18. Zhang, D. X. and G. M. Hewitt. (1998). Field collection: animals in Karp, A., P. G. Isaac and D. S. Ingram (eds.) Molecular Tools for Screening Biodiversity: Plants and Animals. Chapman and Hall. London, Weinheim, New York, Tokyo, Melbourne, Madras. pp 46-48.

7/7/2009