THE USE OF TWO DIFFERENT PRESERVATIVES AND DNA EXTRACTION METHODS FOR TISSUES OF *CRASSOSTREA IREDALEI* (OYSTER) IN PCR AMPLIFICATION STUDY

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FAKULTI SAINS DAN TEKNOLOGI
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2006

The use of two different preservatives and dna extraction methods for tissues of Crassostea Iredalei (Oyster) in pcr amplification study / Kong Hui Jie.

THE USE OF TWO DIFFERENT PRESERVATIVES AND DNA EXTRACTION METHODS FOR TISSUES OF *CRASSOSTREA IREDALEI* (OYSTER) IN PCR AMPLIFICATION STUDY

By

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Research Report submitted in partial fulfilment of
the requirements for the degree of
Bachelor of Science (Biological Sciences)

Department of Biological Sciences
Faculty of Science and Technology
KOLEJ UNIVERSITI SAINS DAN TEKNOLOGI MALAYSIA
2006

This project should be cited as:

Kong, H.J. 2006. The Use of Two Different Preservatives and DNA Extraction Methods for Tissues of *Crassostrea iredalei* (Oyster) in PCR Amplification Study. Undergraduate thesis, Bachelor of Science in Biological Sciences, Faculty of Science and Technology, Kolej Universiti Sains dan Teknologi Malaysia. Terengganu. 48p.

JABATAN SAINS BIOLOGI
FAKULTI SAINS DAN TEKNOLOGI
KOLEJ UNIVERSITI SAINS DAN TEKNOLOGI MALAYSIA

PENGAKUAN DAN PENGESAHAN LAPORAN
PROJEK PENYELIDIKAN I DAN II

Adalah ini diakui dan disahkan bahawa laporan penyelidikan bertajuk: THE USE OF TWO DIFFERENT PRESERVATIVES AND DNA EXTRACTION METHODS FOR TISSUES OF SPECIES *CRASSOSTREA IREDALEI* (TIRAM) IN PCR AMPLIFICATION STUDY oleh Kong Hui Jie, no. matrik: uk 7815 telah diperiksa dan semua pembetulan yang disarankan telah dilakukan. Laporan ini dikemukakan kepada Jabatan Sains Biologi sebagai memenuhi sebahagian daripada keperluan memperolehi Ijazah Sarjana Muda Sains - Sains Biologi, Fakulti Sains dan Teknologi, Kolej Universiti Sains dan Teknologi Malaysia.

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ACKNOWLEDGEMENTS

In the completion of this project, a number of important people have made great contributions and merit special appreciation. My First and foremost appreciation goes to my project supervisor, Cik Wan Bayani Wan Omar, for her guidance and support throughout this project. My special thanks were dedicated to her for being helpful during the sampling period. Her useful comments and suggestions have guided me in completing this project.

I would also like to thank my co-supervisor, Dr. Zaleha binti Kassim that have contributed her ideas in doing this project as well as assistance in identifying species.

I also thank my father, brother and sisters for their continuous moral and financial support, encouragement and prayers. I dedicate my sincere thanks to all my team members for their encouragement, advices and for being kind and helpful.

Above all, I thank my God for granting me much wisdom, knowledge, health and the ability to understand in order that this project may be completed successfully.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
λ	Lambda
%	Percentage
bp	base pair
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediamine tetra-acetic acid
g	Gram
kb	Kilobase
L	Litre
µL	Microlitre
µg	Microgram
mL	Mililitre
mM	Milimolar
OD	Optical Density
rpm	Revolution per minute
SDS	Sodium Dodecyl Sulphate
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TNES-urea buffer	Tris-NaCl-EDTA-SDS-urea buffer
v/v	volume/volume
w/v	weight/volume

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ABSTRACT

Crassostrea iredalei has been known as an important commercial species and have potential for aquaculture. The purity and quality of DNA extracted from tissue samples is important for sensitivity and usefulness of molecular methods such as RAPD-PCR. Therefore, the availability of effective DNA extraction methods is essential. Successful preservation of tissue sample is required for long term molecular studies in distant areas to prevent DNA degradation. In this study, the best preservative and DNA extraction method that produce DNA of highest purity and quality was determined. Two different preservatives were used to preserve the tissue samples and two different DNA extraction methods were used to extract the genomic DNA for PCR amplification. Fresh tissues were used as a control. The purity and quantity of extracted DNA was measured with a spectrophotometer and verified by agarose gel electrophoresis. Finally, the extracted DNA was selected for RAPD-PCR. The purity and quantity of DNA extracted from 95% ethanol was ranged from 1.078 to 1.291 and 260.0 ng/μL to 492.5 ng/μL .The DNA purity and quantity of DNA extracted from TNES-urea buffer was in range of 1.167 to 1.355 and 302.5 ng/μL to 505.0 ng/μL respectively. Based on the banding patterns generated by agarose gel electrophoresis, the Promega Wizard™ Genomic DNA Purification Kit was a good DNA extraction method compared to Phenol-chloroform method and the TNES-urea buffer preservative is a good preservative for *Crassostrea iredalei*.

PENGGUNAAN DUA BAHAN AWET BERLAINAN DAN KAEDAH PENGEKSTRAKAN DNA BERLAINAN BAGI TISU *CRASSOSTREA IREDALEI* (TIRAM) DALAM KAJIAN AMPLIFIKASI PCR

ABSTRACT

Crassostrea iredalei adalah spesies komersial yang penting dan mempunyai potensi untuk akuakultur. Ketulenan DNA yang diekstrak daripada tisu sampel adalah penting bagi kepekaan kaedah molekular seperti RAPD-PCR dalam pemilihan stren *C. iredalei* yang baik. Pengawetan tisu yang baik diperlukan untuk kajian molekular di kawasan yang jauh untuk mengelakkan degredasi DNA. Dalam kajian ini bahan awet dan keadah pengekstrakan DNA yang menghasilkan DNA yang paling tulen dan berkualiti ditentukan. Dua kaedah pengawetan yang berbeza digunakan untuk mengawet tisu dan dua kaedah pemencilan DNA digunakan untuk memencilkan DNA genomik untuk amplifikasi PCR. Ketulenan dan kuantiti DNA ditentukan dengan spektrofotometer dan diverifikasi dengan elektroforesis gel agaros. Akhirnya, DNA yang diekstrak dipilih untuk RAPD-PCR. Ketulenan dan kuantiti DNA yang dipencil daripada tisu dalam pengawet 95% ethanol adalah dalam julat 1.078 hingga 1.291 dan 260 ng/μL hingga 492.5 ng/μL masing-masing, manakala daripada tisu dalam pengawet TNES-urea buffer adalah dalam julat 1.167 hingga 1.355 dan 302.5 ng/μL hingga 505.0 ng/μL. Keputusan berdasarkan corak jaluran elektroforesis menunjukkan kaedah ekstraksi DNA yang paling efisyen adalah Wizard™ Genomic DNA Purification Kit dari Promega manakala penimbal TNES-urea adalah bahan pengawet yang sesuai bagi *Crassostrea iredalei*.

CHAPTER 1

INTRODUCTION

Oysters are mollusks of the class bivalvia which are protected by two permanent shells (Campbell, 2002). Oysters are important as food and numerous countries have been culturing them (Garrido-handog, 1990). In Malaysia, *Crassostrea iredalei* has been known to be an important commercial species due to its sweet flavour and cream coloured meat (Devakie, 1997). This species is commonly served with their shells intact (Devakie, 1997).

Among the important commercial bivalves, *C. iredalei* is mainly farmed by fishermen in Philippines to add to their income other than fishing. On average, oyster farming contributes 30% of the total income of a fisherman (Giselle *et al.*, 1997). One trend in oyster culture in Asia has been the establishment of oyster culture to meet the growing requirement for oyster seed (Garrido-Handog, 1990). The oyster industry in Asian countries are facing several problems and constrains, most of which are related. Pollution on growing sites, lack of oyster seed and lack of waste treatment facilities are major constrains in oyster mariculture (Garrido-Handog, 1990). Oyster culture depends solely on the availability and abundance of wild seed supply (Korringa, 1976).

In Malaysia, oyster culture is not well developed with a current production of about 10 million tones a year (Ng, 1993). This is due to the lack of seeds for oyster culture in this country (Ng, 1993). Large scale production of seeds need to be implemented in

order to meet market demands of *C. iredalei* (Ng, 1993) as the role of oyster culture increases, the demand for genetically improved strains will increase as well. Molecular approaches such as RAPD to screen for genetic relationship and variability among closely related species will therefore aid in the process of selection of better strains (Ng, 1993; Williams *et al.*, 1990).

The development of molecular tools for the identification of genome and genetic variation within species however require pure genomic DNA of high integrity from tissue samples (Ong *et al.*, 1998; Lemarchand *et al.*, 1999). The purity and quality of DNA extracted from tissue samples is therefore an important issue in the sensitivity and usefulness of these molecular methods (Lemarchand *et al.*, 1999). DNA extraction is required for most molecular studies (Susan, 2005). A specific protocol for genomic DNA extraction is required for different species of organism (Brandon *et al.*, 2003). Therefore, the availability of effective DNA extraction methods is essential (Tien *et al.*, 1999).

A freshest preserved tissue sample is required for successful molecular studies, especially for long-term field studies in far areas. DNA should be extracted immediately to prevent degradation. However, most marine biodiversity is centered in the Indo-Pacific, where immediate analysis of DNA is often impossible, thus preservation is required (Dawson *et al.*, 1998). Poor preservation method may result in poor DNA quality. For example, PCR amplification of DNA from poorly preserved tissues may produce poor result. This is because the template molecule of DNA has damaged (Soltis and Soltis, 1993).

DNA analyses are invaluable in studies of the evolution, systematics, and population genetics of marine invertebrates. However, there is lack of information about how best to preserve marine invertebrate tissues for DNA analyses (Chambers *et al.*, 1998). Invertebrate samples may be successfully preserved by using several chemical or physical treatments (Seutin *et al.*, 1991). A preferred method of DNA protection is cryopreservation. This may be accomplished by freezing samples with dry ice at -780°C or in liquid nitrogen at -196°C. However, deep freezing is not always available. Dry ice and liquid nitrogen are both difficult to use in the field because they require careful handling and special equipment. Furthermore, there are strict regulations with limit their transport by air (Dawson *et al.*, 1998).

Since there is lack of information about the optimized DNA extraction method and preservative of *C. iredalei*, this study is done. By optimizing the DNA extraction methods and different preservative for this species, further molecular studies for this species can be done.

Thus, the objectives of this study could be summarized as follows:

i) To measure the purity and quantity of DNA from 95% ethanol and TNES-urea buffer.

ii) To compare the efficiency of Promega Wizard™ Genomic DNA Purification Kit and Phenol-chloroform method from DNA extraction method.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy and Morphology

Oysters are molluscs with a soft unsegmented body protected by two permanent hard shells which increases in size as it grows (Campbell, 2002). They belong to the family Ostreidea which comprises three genera, namely Ostrea, Crassostrea and Pyncnodonta. Examples are the Edible Oyster, *Ostrea edulis* (others are just as edible); the Olympia Oyster *Ostreola conchaphila*; Wellfleet oyster and the Eastern Oyster *Crassostrea virginica*. There are more than 100 known species of oysters, but only several species are widely cultivated (Garrido-handog, 1990). Oysters are highly prized as food, both raw and cooked. They are low in calories, one dozen raw oysters contain approximately 110 calories (460 kJ), rich in iron and high in calcium and vitamin A (The free encyclopedia, 2005).

The lower left shell is usually cupped and upper right shell generally flat. The two shells are roughly textured and are attached by a muscular hinge (adductor muscle) at the narrow end. The mantle generates the shell. This layer of tissue separates the shell from the soft body (Col, 1996). An elastic ligament at the umbonal and anterior end hinges the two shells. The hinge force tends to spring open the two valves, which is opposed by the action of the adductor muscle. The shell is nacreous inside and rough outside. In addition the oyster shell is usually fluted when grown on a hard surface while smooth when grown in muddy bottoms. Salinity levels also affect the shell

structure. Under high salinity conditions, the shells appears hard and the opposite under low salinity conditions (Garrido-handog, 1990).

Internally, the body of the oyster is ventrally and dorsally covered by the mantle, which secretes the shell. The mouth is located towards the umbonal end. Along the ventral part of the body are the gills. On the gill surface are hair-like structures called "cilia", which create an incoming current. The gills and cilia are responsible for collecting food and oxygenation of the blood (Giselle *et al.*, 1997). The digestive system consists of a mouth, oesophagus, stomach, crystalline style, liver and anus, which are located above the adductor muscle. Posterior to adductor muscle is the heart which is very simple, consisting of one auricle and one ventricle. The nervous system is even simpler, being made up of three nerve cells (Giselle *et al.*, 1997). The reproductive organs or gonads are the ovaries in the females and testes in the males, which become greatly enlarged when fully matured (Garrido-handog, 1990). A black marker is usually found in the inner flat shell of *C. iredalei* but not in *C. belcheri*. The meat is whitish in colour in *C. iredalei* while in *C. belcheri*, the meat is brown in colour (Devakie, 1997).

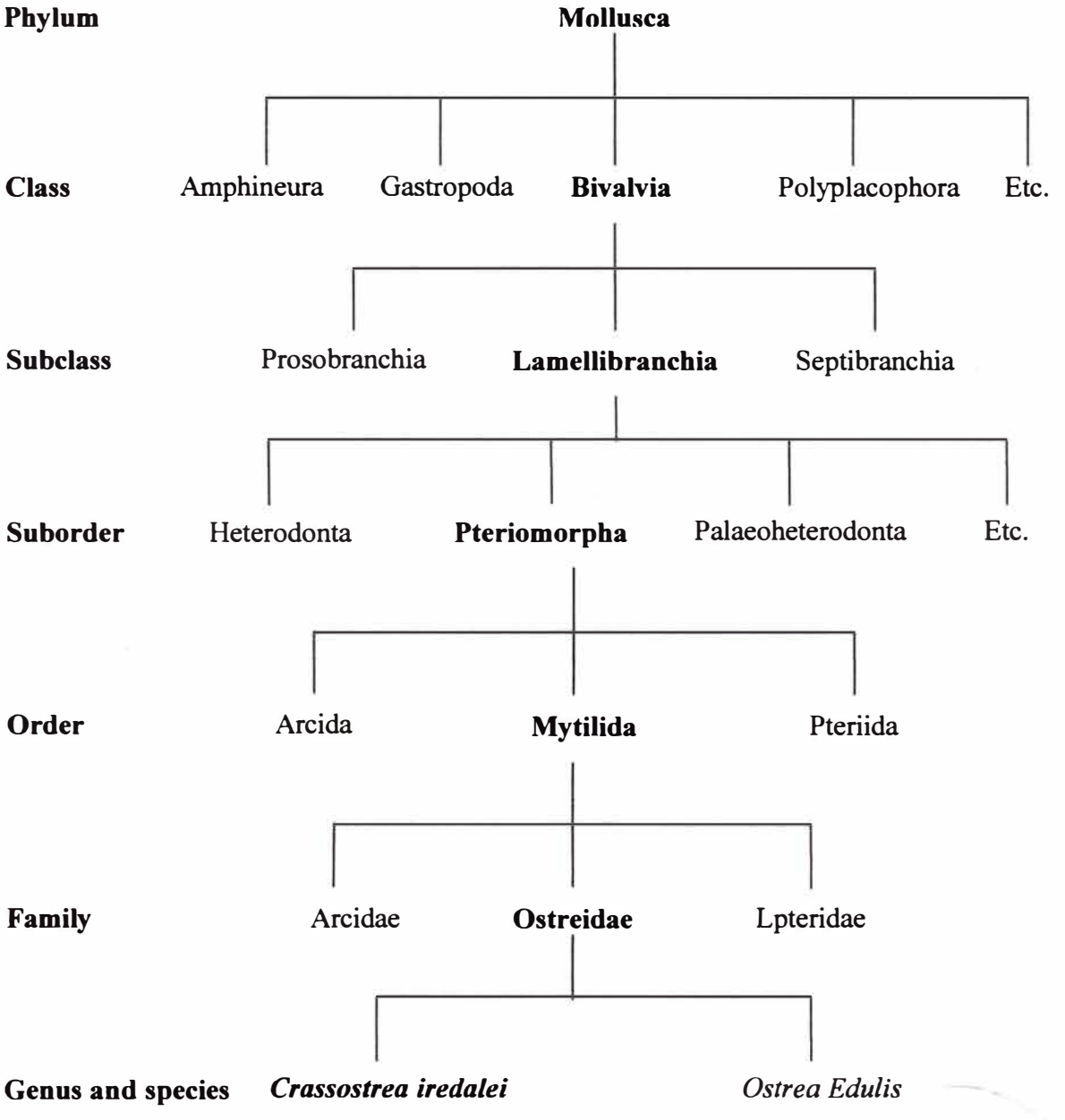
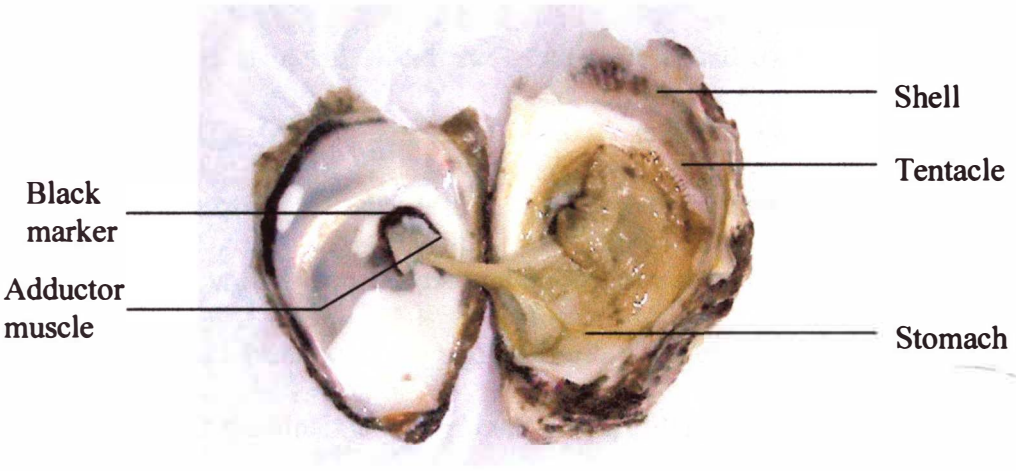


Figure 2.1 The classification of *C. iredalei* (Ross, 2005, and Benjamin, 2004).

(a)



(b)

Figure 2.2 The external and internal view of *C. iredalei*. (a) External view and (b) Internal view of *C. iredalei*.

2.2 Habitat and Distribution

Oysters are widely distributed throughout the world, and inhabit shallow bays and estuarine. Oysters can settle down on any hard surface (Giselle *et al.*, 1997). In the wild, oyster spat can be found on any hard surface like rocks, course sand particles, and roots of mangrove tress, concrete pillars or other permanent structures (Devakie and Ali, 2002). In the absence of suitable substrates, they may even choose to settle on plastic drums, glass pieces, seaweed or shells of other mollusc (Devakie and Ali, 2002).

2.3 Feeding

Oysters feed mainly on phytoplankton, larvae of copepods, protozoa and detritus. In estuaries where the hydrographic conditions are favourable, plankton is abundant and therefore the oysters tend to perform well. During the dry season, the seawater salinity and temperature tend to increase and the oysters are found to be thin and watery (Giselle *et al.*, 1997). Oysters are filter feeders, and are considered obligatory herbivores. Adult oysters are fixed to a hard substrate and therefore the food availability depends entirely on the natural food present in the surrounding water. Thus, oysters completely depend on tidal currents for obtaining food; low current velocities and limited flushing hamper growth (Ross, 2005)

2.4 Reproduction and Growth

Oysters belonging to the genera Ostrea and Crassostrea are quite distinct from each other with regards to their breeding habit (Devakie and Ali, 2002). Ostrea species

exhibit alternation of sexuality within one spawning season. The eggs, after they have been released from the gonad are retained in the mantle cavity while the sperm are extruded externally. The eggs are fertilized by the sperms from outside and half of the larval life takes place in the shell before they are released to the open waters (Ross, 2005). An oyster changes its sex during its life. It starts out as a male and often ends as a female. Oysters of the genus Crassostrea change sex after one spawning season. The sperms and eggs are released into the seawater, either all at one time of small amounts in a long period of time. The eggs are fertilized externally and all subsequent developmental stages occur in the open water (Benjamin, 2004).

2.5 Tissue Preservation

With the increased use of DNA techniques in evolutionary and ecological studies, the methods used for preservation of tissues for DNA extraction are important to protect these potentially valuable resources. The purpose of preservation is to protect the DNA from degradation (Dawson *et al.*, 1998). A preservative is a fluid in which material can be stored for an indefinite period, which without seriously distorting specimens or destroying their constituent parts, arrests autolysis of cells and which also destroys bacteria and moulds (Roger, 1979). According to Roger (1979), most of the aquatic molluscs can be anaesthetized with magnesium sulphate, magnesium chloride, urethane or menthol and with bivalve species good results have been obtained with propylene phenoxetol and phenoxetol BPC.

Several workers (Nietfeldt and Ballinger, 1989; Sibley and Ahlquist, 1981) have suggested storing tissues in ethanol or isopropanol if they are to be used for DNA

extraction. Normally, 95-100% ethanol at ambient temperature is used in preserving tissue sample of invertebrates for molecular studies (Winsor, 1998). Preservation in methanol and propanol is not suitable, although tissue samples preserved in 100% methanol will yield higher molecular weight DNA than samples preserved in formalin (Noguchi *et al.*, 1997). Greer *et al.* (1991) demonstrated that only storage in 95-100% ethanol (v/v) results in PCR products of 1-2 kb after 30 days. Adequate preservation in 70-80% ethanol may occur if specimens or tissue samples are small. Dawson *et al.* (1998) assessed the effects of five buffer solution (70% ethanol, Queen's lysis buffer, DMSO-NaCl solution CTAB-NaCl solution, and a urea extraction buffer) on the preservation of marine invertebrate samples for DNA isolation. They found that the dimethylsulphoxide and sodium chloride (DMSO-NaCl) was the best solution in which to store marine tissue samples.

TNES-urea buffer can be used to preserve vertebrate tissue sample. According to Asahida *et al.* (1996), the vertebrate tissue samples kept up to three years without refrigeration in TNES-urea (6 or 8 M urea; 10 mM Tris-HCl, pH 7.5; 125 mM NaCl; 10 mM EDTA; 1% SDS) result highly yield of molecular weight DNA. A high concentration TNES-urea buffer have been developed suitable for preserving fish muscle and liver samples at ambient temperature for up to three years (Asahida *et al.*, 1996).

2.6 DNA Extraction

The isolation of intact, high-molecular-mass genomic DNA is essential for many molecular biology applications including long PCR, endonuclease restriction

digestion, Southern blot analysis, and genomic library construction (Michiels *et al.*, 2003). There are two or three basic steps in DNA extraction. The cell will be lysed to release the nucleus; the nucleus will be broken down to release DNA. The DNA will be precipitated with alcohol. Cell membranes can be lysed with detergents such as Sodium Dodecyl Sulphate (SDS) (Susan, 2005).

Molecular analysis of the structure and regulation of genes encoding novel, neuron-specific proteins often requires the ability to isolate high quality genomic DNA (Surzycki, 2001). Genomic DNA from tissue samples can be extracted by using available commercial kits such as Promega Wizard™ Genomic DNA Purification Kit or by using manual extraction method such as Phenol-chloroform method. Promega Wizard™ Genomic DNA Purification Kit is an easy and reliable method to purify genomic DNA from neuronal sources as well as whole blood, for which the kit was originally designed. Traditionally, genomic DNA isolations required long and complicated extraction procedures with organic solvents. Promega Wizard™ Genomic DNA Purification Kit now eliminates the hazards and complexity of genomic DNA isolations. The kit provides high yields of genomic DNA with OD_{260}/OD_{280} ratios in the range of 1.6-2.0. The kit isolates high molecular weight DNA, as indicated by comparison to the uncut lambda DNA (Ahmed *et* al. 2003; Surzycki, 2001; Walker *et al.* 2003). The Phenol-chloroform method is the most commonly used method of extracting, purifying and concentrating DNA preparations. This method involves protein removal by a combination of Phenol, chloroform and isoamyl alcohol at a ratio of 25:24:1 and precipitation by ethanol. It is appropriate for the purification of DNA from small volumes (<0.4 mL) at concentrations of < 1 mg/mL (Ausubel *et al.*, 1999).

2.7 PCR Amplification

Polymerase Chain Reaction (PCR) is a method that uses the component of DNA replication to replicate a specific DNA fragment in the test tube (Susan, 2005). This method were developed in 1985, enables a researcher to rapidly isolate a specific DNA without building and screening a library (Susan, 2005). A template molecule and two selected primer are require to start the copying process. Generally, the sequence of the region that flanks the DNA to be amplified must be know, so that primers used in amplification can be synthesized (Susan, 2005). PCR is used to rapidly isolate specific sequences for further analysis or for cloning, identify specific genetic loci for diagnostic or medical purposes, generate DNA fingerprints to determine genetic relationships or to establish identity in forensics and rapidly sequence DNA (Susan, 2005).

PCR is performed by incubation the samples at three temperatures corresponding to the three steps (denaturation, annealing and extension) in a cycle of amplification. The template is denatured by high temperature (94-95°C), then the primers are annealed by lowering the temperature (40-60°C) with *Taq polymerase*, and the DNA polymerase extends the DNA from the primers at70-75°C. It is often helpful to start the first cycle with an initial denaturation step of 3 minutes at 93°C. Specificity can be improved by adding the *Taq polymerase* at a clevated temperature rather than having it present in the reaction prior to the first denaturation step (Innis *et al.*, 1990). About 25-40 cycles are generally conducted using a thermal cycles, and instrument that automatically controls temperature and time. The product generated from the PCR is analyzed by agarose gel electrophoresis (Susan, 2005).

The Random Amplified Polymorphic DNA (RAPD) is a PCR based method; they amplify random DNA sequences which are essentially unknown to the scientist (Susan, 2005). RAPD analysis can be carried out using DNA from an organism for which there is little or no information concerning genomic sequence or organization is known. This technique requires only the presence of a single "randomly chosen' primer (Susan, 2005). The random amplified polymorphic DNA (RAPD)-PCR method allows rapid amplification of DNA fragments to detect genomic polymorphisms. This method utilizes a single short oligonucleotide primer of arbitrary sequence in a PCR (Williams *et al.*, 1993; Williams *et al.*, 1990; McClelland and Welsh, 1994). Unlike restriction fragment length polymorphism RFLP, RAPD-PCR is a simpler, faster, less laborious and inexpensive procedure (Pan *et al.* 1997). RAPD can be used as genetic markers because they are polymorphic. Polymorphism can be simply identified as a result of the presence or absence of discrete amplification products (Susan, 2005). One RAPD primer usually yields more than one marker and the number of RAPD primers available is virtually unlimited, so many markers can be quickly identified (Bickel *et al.*, 1993). This method is often used as a means to characterize different cultivars of crop plants or to identify potential mates for captive animals to maintain or increase genetic diversity (Susan, 2005).

The development of RAPD DNA markers enables estimations of genetic variation between organisms without prior knowledge of sequence information (Williams *et al.*, 1990). One step in developing a map of the equine genome is to establish a foundation physical map (Williams *et al*, 1990). Random amplified polymorphic DNA markers (RAPDs) can be used with a panel of somatic cell hybrids to establish markers (Bickel *et al.*, 1993; Caetano-Anolles *et* al., 1992). RAPD-PCR has been used for genetic

mapping (Welsh and McClelland, 1990; Welsh *et al.*, 1991) population genetics and evolutionary studies (Tibayrenc *et al.*, 1993). Recently, RAPD-PCR was used to detect genomic variability in cancer tissues and human genomic libraries (Ong *et al.*, 1998; Dioh *et al.*, 1997).

RAPD sequences are used to detect variability or polymorphisms in the PCR priming sites. The PCR product will be affected if there are base changes within the target DNA. This will change the DNA fragment profile produced by a specific primer. Usually, about 8 to 10 nucleotide primers are used. These primers are randomly selected with arbitrarily sequences. Sometimes, a computer is used to generate primer. Therefore, in a population of individuals, DNA sequences of various sizes will be amplified with some primers and with others, there may not be a PCR product. Thus, patterns of banding on gels are not the same for every individual in a population (Susan, 2005). The RAPD-PCR technique may be useful for the identification of human molecular markers that may correlate with susceptibility to HIV-1-infection, or differences in disease progression among HIV-1-infected individuals (Felix *et al.* 1998).

CHAPTER 3

MATERIAL AND METHODOLOGY

3.1 Collection of Samples

Fifteen samples of *Crassostrea iredalei* were collected at Setiu Wetland, Terengganu and were stored temporarily in an ice-box.

3.2 Tissue preservation

The adductor muscles of 15 selected individuals were removed and washed with distilled water. Ten selected adductor muscles were preserved separately in 95% ethanol and TNES-urea buffer (Asahida *et al.*, 1996) respectively for three months, while the remaining five samples were frozen at -20°C. The structure and physical properties of the tissues were observed in a period of first, second and third month.

3.3 Genomic DNA extraction

For each preservative method, DNA extraction was carried out every thirty days within three months using Promega Wizard™ Genomic DNA Purification Kit and Phenol-chloroform method. DNA extraction for fresh tissue was carried with both methods as a control.

3.3.1 Genomic DNA Purification Kit (Promega)

Seventy milligrams of adductor muscle tissue was used per extraction in a 1.5 mL microcentrifuge tube. Six hundred microlitres of lysis buffer was added to the adductor muscle. The mixture was homogenized gently until homogenized using a homogenizer. Then, the mixture was incubated at 65°C for 20 minutes. The tube was vortex vigorously to obtain efficient cell lysis. A volume of 3.0 µL RNase will be added and inverted 25 times to mix followed by incubation at 37°C for 15 min. The sample was left at room temperature for 5 min.

Two hundred microlitres of "Protein Precipitation Solution" was added and was vortex for 20 s. After that, the centrifugation was done at 14,000 rpm for 3 minutes at room temperature to precipitate the protein. Seven hundred microlitres of supernatant was transferred to a clean 1.5 mL microcentrifuge tube. After that, 600 µL of isopropanol was added to the supernatant. The solution was inverted until the white thread-like strands of DNA form a visible mass. Then, centrifugation was done for 2 minutes at 14,000 rpm at room temperature. A small white pellet of visible DNA pellet was found at the bottom of the tube. Then, the supernatant was decanted and 600 µL of room temperature 70% ethanol was added. The tube was gently inverted for several times to wash the DNA pellet. After that, centrifugation was done for 1 minute at 14,000 rpm at room temperature and the ethanol was decanted. The tube was inverted on a clean absorbent paper and the pellet was air-dried for 10-15 minutes. A volume of 100 µL of DNA rehydration buffer was added to the tube and was incubated at 65°C for 1 hour. Periodically the solution was mixed by gently tapping

the tube. Alternatively, the DNA was rehydrated by incubating the solution overnight at room temperature. Lastly, the genomic DNA will be stored at -20 °C.

3.3.2 Phenol-Chloroform Method

DNA was extracted based on the Phenol-chloroform protocol with minor modifications. First, 500 µL of digestion buffer {1% (w/v) SDS, 0.8% (v/v) Triton X-100, 0.5 M NaCl, 0.1 M Tris-HCl at pH 9, 0.01 M EDTA} was added into 1.5 mL microcentrifuge tube which contained 70 mg adductor muscle tissue. Then, 40 µL of 10% SDS and 40 µL of Proteinase K were added. The tube was shaken gently and was incubated for 1-2 hours at 55°C. Then, 25 µL of RNase was added and was inverted for 20 times before incubated at room temperature for 15-30 minutes. The mixture was vortex once every 15 minutes during incubation. After that, 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The mixture was inverted for 30 times and vortex gently to homogenize. Then, centrifugation was done at 13,000 rpm for 5 minutes. Then, the top aqueous layer was removed and was dispensed into a new microcentrifuge tube. The step of adding phenol:chloroform:isoamyl alcohol (25:24:1) was repeated. After that, 500 µL of chloroform:isoamyl alcohol (24:1) was added and the mixture was centrifuged at 13,000 rpm for 5 minutes. The top aqueous layer was transferred into new microcentrifuge tube.

After that, 1 mL of ice-cold absolute ethanol was added to the upper aqueous layer and the mixture was inverted rapidly for several times. Then, centrifugation was performed at 6,000 rpm for 30 minutes. Precipitated DNA was collected at the bottom

of the tubes as white pellets. The pellets were washed with 500 µL of 70% ethanol followed by centrifugation at 6,000 rpm for 30 minutes. The DNA was dried at room temperature. Then, 100 µL of TE buffer (10 mM Tris and 1 mM EDTA, pH 8) was added and left for at least 24 hours at room temperature to dissolve the pellet completely before proceeding to the next step. Finally, the DNA extraction sample was kept in -20°C to avoid DNA degradation.

3.4 Analysis

3.4.1 Analysis of Genomic DNA Quality by Agarose Gel Electrophoresis

Genomic DNA was separated by gel electrophoresis through 1.0% agarose gel. The gel was prepared by mixing 1.0 g of agarose with 100 mL of 1.0X TBE (10 mM Tris, 1 mM EDTA pH 8.0) in a 500 mL flask. The mixture was boiled for 3 minutes in a microwave oven to dissolve the agarose. Then, 1 µL ethidium bromide (0.5 µg/mL) was added and mixed gently. The mixture was poured into a mould which was fitted with a well-fitting comb and was left to at room temperature until it solidifies (20-30 minutes). The the gel casting combs were carefully removed and the gel was placed in a horizontal electrophoresis apparatus.

A volume of 10 µL genomic DNA from each sample was mixed with 2.5 µL of loading dye, and was load on the agarose gel. Then, 1 kb ladder marker (λ *Hind*III DNA molecular weight marker) was added into the first lane on the gel. Electrophoresis was carried out at 75 volts for 1 hour. The gel was washed with distilled water for 5-10 minutes prior to photographing with Image Master VDS. The

genomic DNA with the sharpest and clearest band of fresh tissue and each preservative was selected for RAPD-PCR.

3.4.2 Measurement of DNA Purity and Quantity

Purity and quantity of the genomic DNA was estimated using UV-VIS Spectrophotomer. A volume of 10 µL genomic DNA from each sample was dissolved in 490 µL of TE buffer in a 0.5 mL cuvette and mixed. The absorbance reading at 260nm (OD_{260}) and 280nm (OD_{280}) of genomic DNA was measured and recorded. The purity of DNA was estimated by calculating the ratio of absorbance readings at 260nm and 280nm (OD_{260}/OD280). An OD_{260}/OD_{280} ratio of 1.8 is characteristic of pure DNA. An OD_{260}/OD_{280} ratio of 2.0 is characteristic of pure RNA. A pure double stranded DNA sample has an absorbance of 1.0 at 260nm. It also contains approximately 50 µg/mL of double stranded DNA. The DNA concentration will be determined by the formula:

DNA concentration = OD_{260} X 50 µg/mL dilution factor

3.4.3 DNA Amplification of RAPD Primers

Each selected genomic DNA was amplified with 10 primers (OPA-01 – OPA-10) with 60-70% GC content by PCR. A total reaction volume of 25 µL was used with the final concentration containing 1 X of reaction buffer including 50 ng of genomic DNA, magnesium chloride (3.0mM), Taq DNA Polymerase (2 Units), dNTP-mixture (0.4 mM) and primer (10 picomoles). The PCR conditions that was carried out were; 45 cycles for 30 seconds at 94°C to denature the DNA strand, 30 seconds at 36°C to

anneal the primers, 1 minute at 72°C to extend the PCR products and a final extension of 2 minutes at 72°C.

Amplified products were analyzed in 1.5% agarose gel electrophoresis at 55 volts for 1-2 hours and photographed with VDS-image master. The 100 ladder plus marker was used as a molecular weight standard. Each set of PCR products included negative control to ensure that the observed banding patterns was reproducible, repeatable and uncontaminated. The electrophoresis product for each sample was compared.

Table 3.1 Code, sequence, nucleotide length and G+C content of primers used in Random Amplified Polymorphic DNA analysis.

No	Primer code	Primer sequence 5' to 3'	Nucleotide Length	G+C Content (%)
1	OPA-01	CAGGCCCTTC	10-mer	70.0
2	OPA-02	TGCCGAGCTG	10-mer	70.0
3	OPA-03	AGTCAGCCAC	10-mer	60.0
4	OPA-04	AATCGGGCTG	10-mer	60.0
5	OPA-05	AGGGGTCTTG	10-mer	60.0
6	OPA-06	GGTCCCTGAC	10-mer	70.0
7	OPA-07	GAAACGGGTG	10-mer	60.0
8	OPA-08	GTGACGTAGG	10-mer	60.0
9	OPA-09	GGGTAACGCC	10-mer	70.0
10	OPA-10	GTGATCGCAG	10-mer	60.0

CHAPTER 4

RESULTS

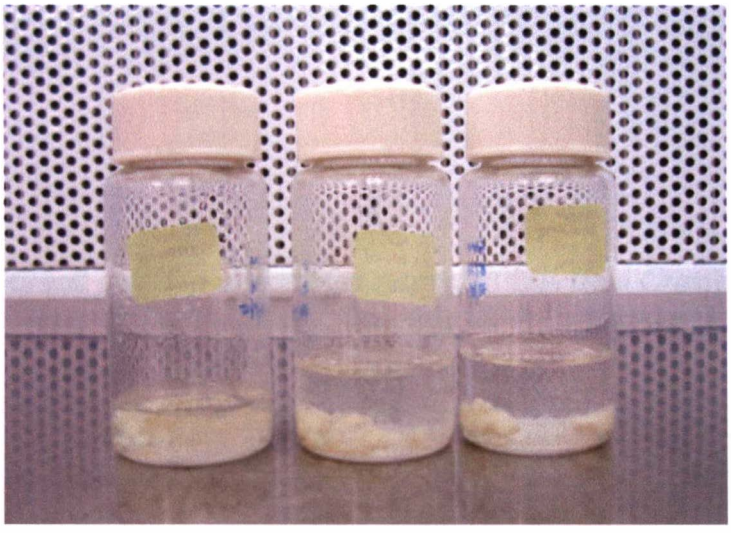
4.1 Structure and Physical Properties of Tissues

The structure and physical properties of tissues that were preserved in 95% ethanol and TNES-urea buffer were observed in a period of first, second and third month. The tissue preserved in 95% ethanol shrunk, hardened changed to white in the first second and third month while the tissue preserved in TNES-urea buffer began to loss its opacity in the first month and dissolved within two months.

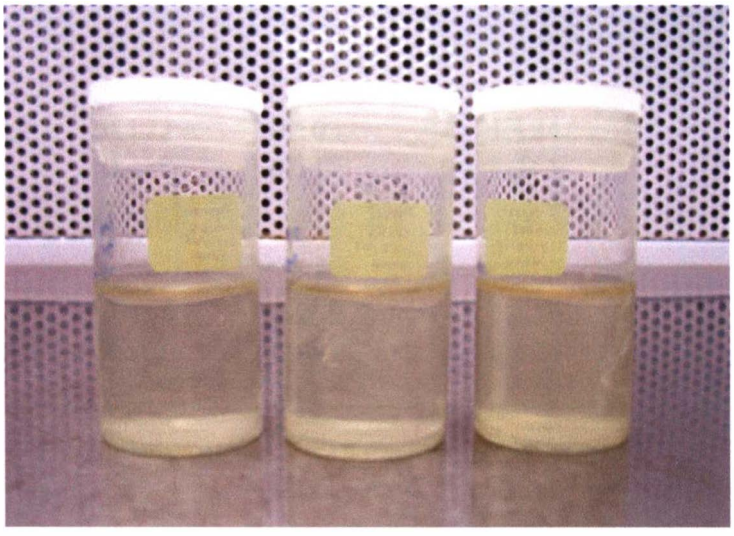
4.2 Purity and Quantity of Genomic DNA

The Purity and quantity of genomic DNA which was extracted from fresh tissue and tissue preserved in both 95% ethanol and TNES-urea buffer was measured using a spectrophotometer at OD_{260} and OD_{280} as shown in Table 4.1 and 4.2. The purity and quantity of genomic DNA from fresh tissue extracted with Promega Wizard™ Genomic DNA Purification Kit was 1.291 and 432.5 ng/μL. The purity and quantity of DNA extracted for *C. iredalei* with Promega Wizard™ Genomic DNA Purification Kit from 95% ethanol was in range of 1.130 to 1.279 and 260.0 ng/μL to 492.5 ng/μL; from TNES-urea buffer was ranged from 1.078 to 1.165 and 260.0 ng/μL to 412.5 ng/μL (Table 4.1).

For the Phenol-chloroform method, the purity and quantity of genomic DNA from fresh tissue was 1.189 and 302.5 ng/μL. The DNA purity and quantity was in range of 1.340 to 1.355 and 342.5 ng/μL to 477.5 ng/μL for 95% ethanol. Meanwhile the purity and quantity of DNA from TNES-urea buffer was in range of 1.167 to 1.202 and 490.0 ng/μL to 505.0 ng/μL respectively (Table 4.2).



(a)



(b)

Figure 4.1 Tissues of *Crassostrea iredalei* preserved in 95% ethanol and TNES-urea buffer. (a) Tissue preserved in 95% ethanol shrunk, hardened changed to white; (b) tissue preserved in TNES-urea buffer began to loss its opacity.

Table 4.1 Purity and quantity of extracted genomic DNA from *Crassostrea iredalei* with Kit (Promega Wizard™ Genomic DNA Purification Kit).

Sample	OD_{260}	OD_{280}	Ratio/Purity of Genomic DNA (OD_{260}/ OD_{280})	DNA Concentration/ Quantity of Genomic DNA (ng/µl)
Fresh Tissue	0.173	0.134	1.291	432.5
1st month				
95% ethanol	0.197	0.154	1.279	492.5
TNES-urea buffer	0.165	0.153	1.078	412.5
2nd month				
95% ethanol	0.122	0.103	1.184	305.0
TNES-urea buffer	0.130	0.112	1.161	325.0
3rd month				
95% ethanol	0.104	0.092	1.130	260.0
TNES-urea buffer	0.106	0.091	1.165	265.0

Table 4.2 Purity and quantity of extracted genomic DNA from *Crassostrea iredalei* with Phenol-chloroform method.

Sample	OD_{260}	OD_{280}	Ratio/Purity of DNA (OD_{260}/ OD_{280})	DNA Concentration/ Quantity of DNA (ng/µl)
Fresh Tissue	0.121	0.101	1.198	302.5
1st month				
95% ethanol	0.148	0.110	1.345	370.0
TNES-urea buffer	0.196	0.168	1.167	490.0
2nd month				
95% ethanol	0.191	0.141	1.355	477.5
TNES-urea buffer	0.200	0.170	1.176	500.0
3rd month				
95% ethanol	0.137	0.102	1.340	342.5
TNES-urea buffer	0.202	0.168	1.202	505.0

4.3 DNA Extraction

The quality and integrity of the DNA tissue was verified with 1.0% of agarose gel electrophoresis. In Figure 4.1 and Figure 4.2, clear distinct bands of about 23 Kb with minimum smearing was obtained, thus DNA from lane 5 and 6 from Figure 4.1 was selected for PCR amplification. Lane 1 was amplified as control (Figure 4.1). The clear bands produced by genomic DNA as verified by agarose gel electrophoresis shows that the DNA was high in quality.

4.4 PCR Amplification

The genomic DNA of highest quality and integrity (clearest band) extracted with Promega Wizard™ Genomic DNA Purification Kit from tissue preserved in 95% ethanol, TNES-urea buffer and fresh tissue (as control) were selected for RAPD-PCR. Ten primers (OPA-01 to OPA-10) with 60-70% GC content were chosen for PCR amplification. All lanes except lane 6 and 7 for fresh tissue (Figure 4.3), lane 6 for tissue preserved in 95% ethanol (Figure 4.4) and lane 5 and 6 (Figure 4.5) showed clear bands.

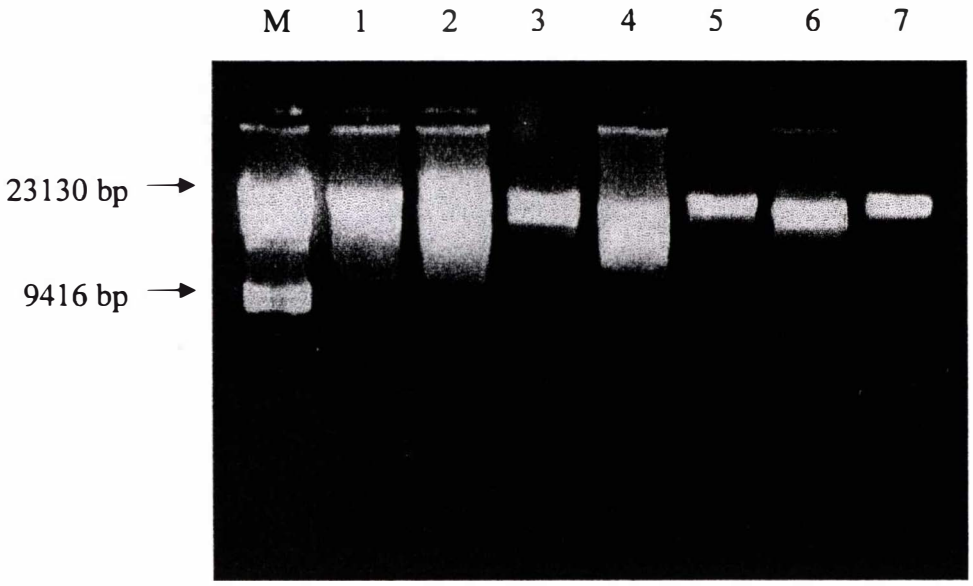


Figure 4.2 Genomic DNA extracted using Promega Wizard™ Genomic DNA Purification Kit. M represents the λ *Hind*III marker. A clear band of about 2.3 Kb was obtained with minimum smearing. Fresh tissue (Lane 1), Ethanol; 1st, 2nd and 3rd month (Lane 2, 4, 6), TNES-urea buffer; 1st, 2nd and 3rd month (Lane 3, 5, 7)

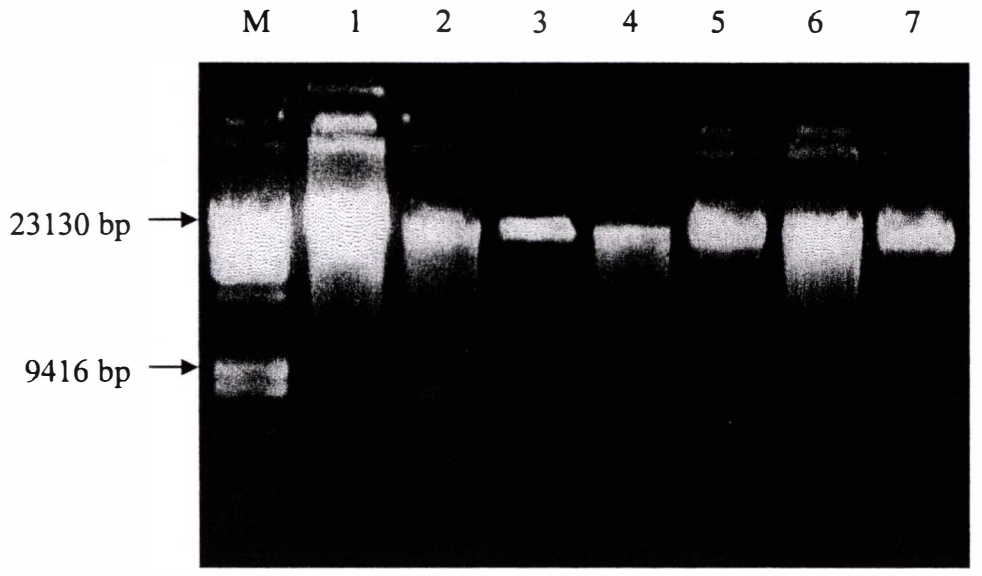


Figure4.3 Genomic DNA extracted using Phenol-chloroform method. M represents the λ *Hind*III marker. A clear band of about 2.3 Kb was obtained with minimum smearing. Fresh tissue (Lane 1), Ethanol; 1st, 2nd and 3rd month (Lane 2, 4, 6), TNES-urea buffer; 1st, 2nd and 3rd month (Lane 3, 5, 7)

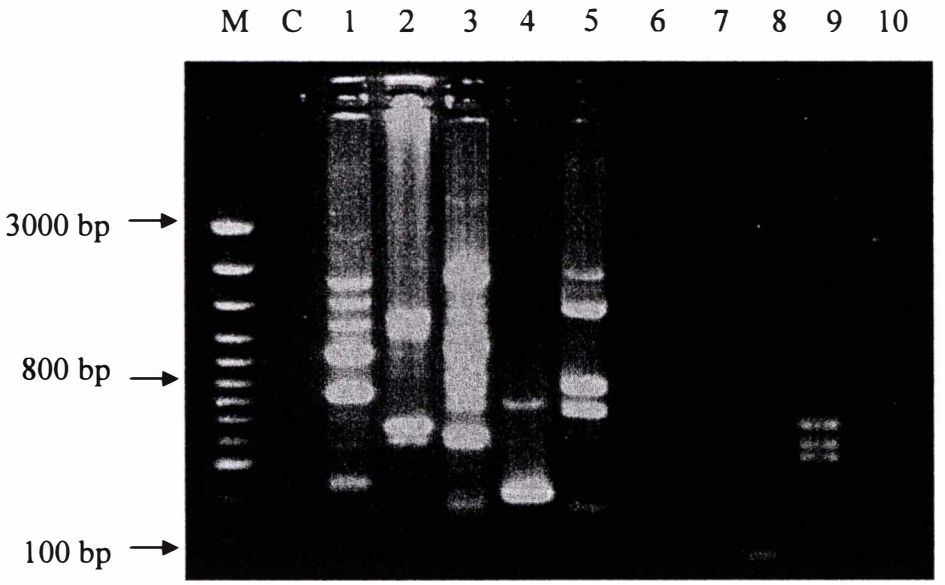


Figure 4.4 RAPD banding patterns for RAPD-PCR from fresh tissue generated by 10 primers; OPA01- OPA10 (Lane 1- lane 10). The genomic DNA was extracted by using Promega Wizard™ Genomic DNA Purification Kit. M represents the 100 bp ladder plus marker and C represents negative control.

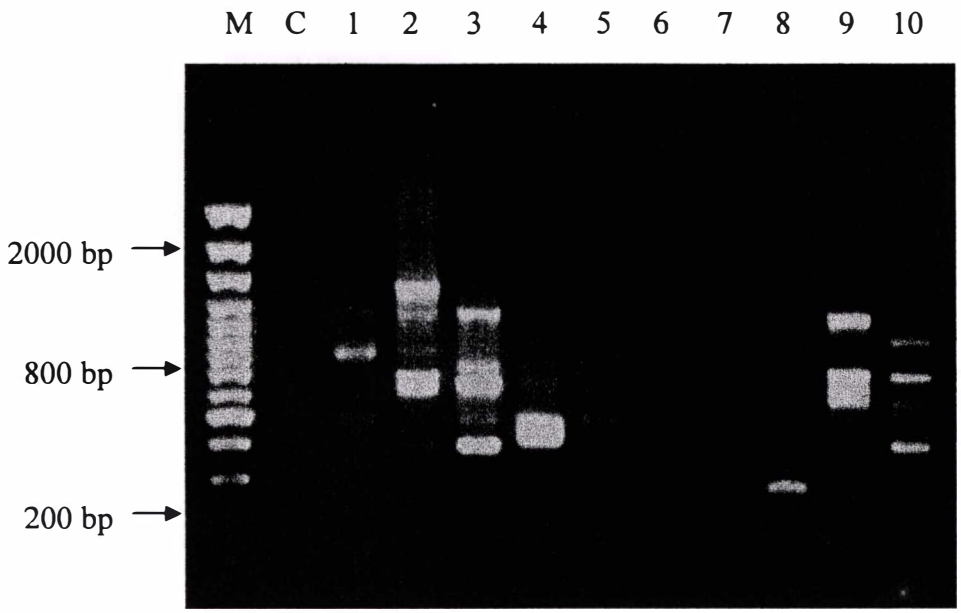


Figure 4.5 RAPD banding patterns for RAPD-PCR from tissues in 95% ethanol generated by 10 primers; OPA01- OPA10 (Lane 1- lane 10). The genomic DNA used was extracted by using Promega Wizard™ Genomic DNA Purification Kit. M represents the 100 bp ladder plus marker and C represents the control.

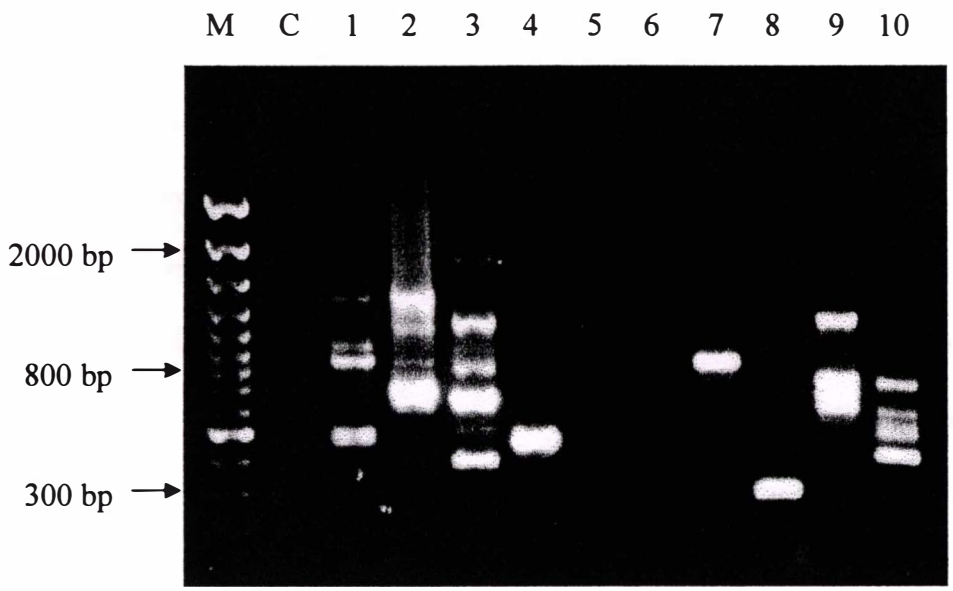


Figure 4.6 RAPD banding patterns for RAPD-PCR from tissue in TNES-urea buffer generated by 10 primers; OPA01- OPA10 (Lane 1- lane 10). The genomic DNA used was extracted by using Promega Wizard™ Genomic DNA Purification Kit. M represents the 100 bp ladder plus marker and C represents the control.

CHAPTER 5

DISCUSSION

5.1 Structure and Physical Properties of Preserved Tissue

The type of tissue, the chemical and physical environment in which that tissue is stored, and the duration of storage will affect the preservation of DNA (Dawson *et al.*, 1998). Tissue type has also been found to affect the success of preservation and DNA analyses of samples from plants (Pyle and Adams, 1989). In this study, muscle tissue was chosen for two reasons. First, muscle tissue was chosen to avoid contamination from impurities such as enzymes from stomach. Secondly, the dissection from the muscle is easier to extract a sufficient amount of tissue than internal organ (Surzycki, 2001). It was reported that the abdomen of some ants must be discarded prior to preservation else the formic acid therein will depurinate the DNA (Altschmied *et al.* 1997).

In this study, the structure and physical properties of tissues that were preserved in 95% ethanol and TNES-urea buffer were observed in a period of first, second and third month. The tissue preserved in 95% ethanol shrunk, hardened and changed to white in the first second and third month (Figure 4.1) while the tissue preserved in TNES-urea buffer began to loss its opacity in the first month and dissolved within two months (Figure 4.1). According to Dawson *et al.* (1998), storage in ethanol dehydrates the sample and results in the denaturation and precipitation of proteins, including catabolic enzymes. In the experiment conducted by Dowson *et al.* (1998) with tissue

from marine invertebrates, physical structure was preserved best by DMSO-NaCl and 70% ethanol. Almost without exception structure could be easily identified after 28 months (Dowson *et al.*, 1998). In contrast, storage in urea resulted in complete dissolution of most samples within 28 months. DMSO-NaCl was the best solution to store marine tissue samples (Dawson *et al.*, 1998). However, according to Dawson *et al.* (1998), the physical condition of a sample is a poor indicator of the quality of DNA in that sample. High molecular weight DNA (~20 kb) was extracted from samples stored for up to 28 months in urea extraction buffer (Dawson *et al.*, 1998).

Preservation of tissue may be facilitated by finely dicing tissue to increase permeation of the storage solution into the sample (Seutin *et al.*, 1991; Reiss *et al.*, 1995). Dessauer *et al.* (1995) suggested that tissue should be minced into pieces less than one mm^3. However, none of the samples that yielded high molecular weight DNA contained pieces less than one mm^3, with the exception of gastric filaments and gonads of *Aurelia* (Dawson *et al.*, 1998).

Duration of storage have effects on degradation of samples over the time. It was reported that the quantity and quality of DNA recovered from samples progressively declines as the duration of storage increases (Post *et al.*, 1993). Visual inspection of samples stored for one, six, and twenty eight months suggests that the greater the duration of storage prior to analysis, the greater the degradation of the sample (Dawson *et al.*, 1998). This pattern was most obvious from the visual assessments of physical quality but was also reflected in increasing fragmentation of high molecular weight DNA with time (Dawson *et al.*, 1998). However, Dawson *et al.* (1998) suggested that the urea-based solution was a suitable preservative suitable for

longterm storage of DNA. TNES-urea buffer is a urea-based preservative that was found to be suitable for long-term storage of tissues for DNA analyses by Asahida *et al.* (1996). In this present study, there were no significant differences in the purity and quantity of DNA from tissue preserved in one, two and three months.

5.2 Purity and Quantity of Genomic DNA

Two different extraction procedures were examined to check their relative efficiencies in extracting DNA from two different preserved tissue of *C. iredalei*. The genomic DNA was measured at two wavelengths at 260nm and 280nm. The extracted genomic DNA was considered pure when the ratio of OD_{260}/OD_{280} of DNA sample was in the range of 1.7 to 2.0 (Sambrook, 2003). A ratio of 1.7 to 2.0 is therefore desired when the OD was taken.

In this study, the results shows that the genomic DNA for *C. iredalei* have poor purity because the overall purity was in the range of 1.078 to 1.355 (OD_{260}/OD_{280}) based on Table 4.1 and Table 4.2. This happened due to the improper mixing of the genomic DNA during measurement with a spectrophotometer. A ratio less than 1.7 means there is probably a contaminant in the solution, typically either protein or Phenol (Sambrook *et al.*, 1988). According to Surzycki (2001), the quantity, quality and integrity of DNA will directly affect the results. However, based on the ratio obtained in this present study, it was found that somehow the samples below this range were not found to have much influence on the banding pattern of genomic DNA and RAPD-PCR products based on the results obtained in Figure 4.2 to Figure 4.6.

In this study, two different methods which were the Phenol-chloroform method and Promega Wizard™ Genomic DNA Purification Kit were used to extract the DNA of *Crassostrea iredalei*. The quantity of genomic DNA extracted with kit was in the range of 260.0 ng/μL to 492.5 ng/μl (Table 4.1) while the quantity of DNA was in range of 302.0 ng/μl to 502.0 ng/μl when extracted using the Phenol-chloroform method (Table 4.2). According to Taggart (1992), variation in extraction procedures and reagents can all produce different samples at different concentration such as Phenol-based protocols.

The purity and quantity of genomic DNA from tissue preserved in 95% ethanol was ranged from 1.130 to 1.335 and 260.0 ng/μL to 492.5 ng/μL respectively; while the purity and quantity of genomic DNA from tissue preserved in TNES-urea buffer was ranged from 1.078 to 1.202 and 265.0 ng/μL to 505.0 ng/μL. The yield and purity of DNA is an important characteristic of these methods. While purity of DNA often is estimated by the ratio of its absorbance at 260nm and 280nm, the unreliability of this method has been pointed out (Shimelis *et al.*, 2004). HPLC has played a role in assessing both the amount and purity of DNA, based on analysis of the deoxynucleotide, or deoxyribonucleoside products formed by subjecting DNA to enzymatic hydrolysis (Shimelis *et al.*, 2004).

5.3 DNA Extraction

In this present study, the genomic DNA of *C. iredalei* was successfully extracted from the muscle tissues. Clear bands with minimum degradation were observed in Figure 4.2 and Figure 4.3. Compared to the Phenol-chloroform method, the genomic DNA of

C. iredalei extracted using Promega Wizard™ Genomic DNA Purification Kit had less degradation. According to Surzycki (2001), Promega Wizard™ Genomic DNA Purification Kit is designed for isolation of DNA from white blood cells, tissue culture cells and animal tissue, plant tissue, yeast, and Gram positive and Gram negative bacteria. The use of this method has been proven in a wide range of organisms. Besides, DNA without major contamination of protein and RNA can be isolated by using this method (Surzycki, 2001). DNA purified by this system is suitable for a variety of applications including amplification, digestion with restriction endonucleases and membrane hybridization. A successful genomic DNA extraction was obtained for the areolated grouper, *Epinephelus areolatur* using this method (Walker *et al.* 2003). The Phenol-chloroform method procedure utilizes many dangerous reagents (Barbaro *et al.* 2004). It is also time consuming since it requires many steps and a particular accuracy to avoid the loss of material (Barbaro *et al.* 2004). Lemarchand *et al.* (2005) found that bead beating separation and Phenol-chloroform-isoamyl alcohol extraction result in the highest yield of DNA but had a medium degradation level and an average purity level.

In this present study, DNA extracted from TNES-urea buffer produce clearer bands compared to 95% ethanol due to the DNA protecting properties of EDTA. According to Kilpatrick (2001), the amount of degradation, as indicated by low molecular weight DNA fragments, was greatest in tissue stored in ethanol. Degradation observed in ethanol preserved tissues occurs during the extraction procedure (Kilpatrick, 2001). Tissue preserved in ethanol yielded relatively large amounts of low molecular weight DNA. Several articles (Houde and Braun, 1988; Seutin *et al.*, 1991) have also concluded that tissues preserved in ethanol yielded primarily highly degraded DNA

fragments. Storage of tissues in either DMSO-salt solution or lysis buffer (both containing EDTA) appears to protect the DNA from degradation during the extraction process, whereas tissue preserved in ethanol (without EDTA) yielded relatively large amounts of low molecular weight DNA (Kilpatrick, 2001). There is a slight degradation of DNA extracted from fresh tissue with both DNA extraction methods due the duration of transport and improper of storage in -20°C.

5.4 PCR Amplification

The quality of the extracted DNA in this study was subsequently assessed by PCR amplification. DNA for PCR amplification was selected based on the clarity and the amount of smearing. DNA from lane 5 and 6 showed a clear and distinct band of about 23 Kb with minimum smearing and thus was selected for PCR amplification (Figure 4.2). DNA from Lane 1 was amplified as positive control (Figure 4.2). In this present study, ten primers (OPA-01 to OPA-10) with 60-70% GC content were chosen for PCR amplification. Most of the primers successfully amplified the DNA except for primers OPA-6 and OPA-7 (Figure 4.4) primer OPA-6 (Figure 4.5); OPA-5 and OPA-6 (Figure 4.6).

Lemarchand *et al.*, (2005) observed differences of extraction efficiencies and differences in PCR outcome between the different extraction protocols. In the experiment conducted by Lemarchand *et al.* (2005), ten different methods of DNA extraction for bacteria were compared. The main difference was observed for DNA extracted by triton-prep method where no amplification was generated by any primer set. Lemarchand *et al.* (2005) concluded that the best method to extract bacterial DNA

was the bead beating separation and ammonium acetate purification. However in the present study, DNA extracted from 95% ethanol and TNES-urea buffer produce clear and reproducible band from RAPD-PCR similar to fresh tissue. The results shows that both DNA extracted from two different preserved tissues were in high quality and purity.

The purity of DNA extracted from tissue samples is an important issue in the sensitivity and the usefulness of molecular methods such as PCR (Lemarchand *et al.* 2005). Although high molecular weight DNA is preferred for PCR, degraded DNAs will often amplify if the target fragment is small enough and primer specific (Pan *et al.* 1997).

CHAPTER 6

CONCLUSION

In conclusion, DNA extracted from *Crassostrea iredalei* preserved in two different preservative with two different methods have a lower OD_{260}/OD_{280} ratio. However, DNA extracted with Promega Wizard™ Genomic DNA Purification Kit produce higher quality DNA as verified by agarose gel electrophoresis. This study shows that tissue stored in TNES-urea buffer yielded higher molecular weight DNA compared to ethanol. DNA extracted from two different preservative with Promega Wizard™ Genomic DNA Purification Kit produced satisfactory result in RAPD-PCR. TNES-urea buffer is a good preservative that suitable for preservation of DNA. However this buffer is not preferred for tissue preservation. Through this study, further studies on oyster molecular genetics and oyster culture in the future can be improved, especially in tissue and DNA preservation methods of oysters.

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APPENDICES

Appendix A

Buffer and Solution

a) Tris-Borate-acid EDTA (TBE buffer, 10X) in 1000 ml at pH 8.0

109 g Tris-base
55 g Boric acid
9.3 EDTA
(Distilled water to the final volume of 1000 ml, autoclave the solution)

b) Tris-EDTA (TE buffer) in 100 ml at pH 8.0

10 mM Tris-HCl
1 mM EDTA
(Distilled water to the final volume of 100 ml)

c) Tris-NaCl-EDTA-SDS-urea buffer (TNES-urea buffer) in 1000 ml at pH 8.0

	for 200 ml	final concentration
Tris	2 ml of 1M pH 7.5	10 mM
NaCl	5 ml of 5 M	125 mM
EDTA-2Na	2 ml of 0.5 M pH 7.5	10 mM
SDS	10 ml of 10%	0.5%
Urea	48.05 g	4 M

(Distilled water to the final volume of 1000 mL)

d) Phenol:Chloroform: Isoamyl-alchohol (25:24:1)

Saturated Phenol : 25 ml
Chloroform : 24 ml
Isoamyl-alcohol : 1 ml

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