Preliminary Molecular Work on Hard Clam, *Meretrix lyrata* from Buntal Bay, Sarawak

WONG FONG FUN

This project is submitted in partial fulfillment requirement for the degree of Bachelor of Science with Honours (Aquatic Resource Science and Management)

Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2009
DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution or higher learning.

_________________________________

Wong Fong Fun

Aquatic Resource Science and Management Program
Department of Aquatic Science
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
ACKNOWLEDGEMENT

Firstly, I would like to thank to my supervisor, Dr. Ruhana Hassan for all her wonderful insight and supervision throughout the duration of this project. I also grateful for guidance and assistance of post graduate students especially Ms. Florence Laiping, Ms. Nur Sara Shahira bt Abdullah and lab assistant, Mr. Richard Toh in completing this project. Besides from that, I would like to thank to my fellow friends especially Amelia Shoon Wan Chin, Mohd Fazhan bin Mohd Hanafiah, Nurhartini Kamalia Yahya and Raymie bin Nurhasan and entire final year students of Department of Aquatic Science for their continuous help and supports in making this project a success. Last but not least, my heartiest gratitude goes to my parents Mr. Wong Ching Sin and Madam Lu Siew Geok and the rest of my family members for their amazing love and support in accomplishing this project.
# Table of Contents

Acknowledgement ............................................................................................................... I

Table of Contents ................................................................................................................ II

List of Abbreviations .......................................................................................................... IV

List of Tables ......................................................................................................................... V

List of Figures ....................................................................................................................... VI

Abstract ............................................................................................................................. 1

Introduction........................................................................................................................... 2

Literature Review .................................................................................................................. 4

  Morphology, Ecology and Importance ............................................................................. 4
  Total Genomic DNA Extraction ....................................................................................... 6
  Polymerase Reactions Chain ............................................................................................. 7
  Gene Of Interest ................................................................................................................ 9
    ITS Region ...................................................................................................................... 9
    Mitochondrial Large Ribosomal Subunit (16S) ............................................................. 12
  PCR-Restriction Fragment Length Polymerase ................................................................. 14

Materials and Method.......................................................................................................... 16

  Field Sampling .................................................................................................................. 16
  Morphology Assessment ................................................................................................. 16
  Total genomic DNA extraction ....................................................................................... 16
    CTAB-chloroform DNA extraction ................................................................................. 16
    Phenol-chloroform DNA Extraction ............................................................................. 17
  PCR amplification ............................................................................................................ 18
    16S rRNA amplification ............................................................................................... 18
    ITS gene region Amplification ...................................................................................... 19
    Cytochrome Oxidase I (COI) gene Amplification ......................................................... 20
  Purification ....................................................................................................................... 21
  Sequencing ....................................................................................................................... 21
  PCR-RFLP Analysis ......................................................................................................... 22
  Sequenced Data Analysis ............................................................................................... 22

Results and Discussion ....................................................................................................... 24

  Field Sampling ................................................................................................................ 24
  Morphology Assessment ................................................................................................. 25
  Total Genomic DNA ....................................................................................................... 28
  Spectrophotometer Reading ......................................................................................... 31
<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymerase Chain Reaction (PCR)</td>
<td>33</td>
</tr>
<tr>
<td>ITS Region Amplification</td>
<td>33</td>
</tr>
<tr>
<td>16S rRNA Amplification</td>
<td>35</td>
</tr>
<tr>
<td>COI Region</td>
<td>38</td>
</tr>
<tr>
<td>PCR Product Purification</td>
<td>41</td>
</tr>
<tr>
<td>PCR-RFLP</td>
<td>42</td>
</tr>
<tr>
<td>Sequence Analysis</td>
<td>46</td>
</tr>
<tr>
<td>16S rRNA gene</td>
<td>46</td>
</tr>
<tr>
<td>ITS region</td>
<td>47</td>
</tr>
<tr>
<td>Phylogenetic analysis</td>
<td>52</td>
</tr>
<tr>
<td>Conclusion and Recommendation</td>
<td>54</td>
</tr>
<tr>
<td>References</td>
<td>56</td>
</tr>
<tr>
<td>Appendix</td>
<td>59</td>
</tr>
</tbody>
</table>
List of abbreviation

μl  Micro liter  
rpm  Round per minutes  
mm  Milimeter  
ml  Mililiter  
mM  Miliimole  
Bp  Base pair  
EtOH  Ethanol  
NaCl  Sodium Chloride  
ddH₂O  Double Distilled Water  
dNTP  Deoxynucleotide triphosphatete  
MgCl₂  Magnesium Chloride  
CTAB  Cetyl Trimethyl Ammonium Bromide  
PCR  Polymerase Chain Reaction  
RFLPs  Restriction Fragments Length Polymorphisms  
mtDNA  Mitochondrial
List of Tables

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1</td>
<td>Sequences of primer used to amplify 16S rRNA</td>
<td>19</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Sequences of primer used to amplify ITS region.</td>
<td>20</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>The restriction enzyme and their cleavages sites</td>
<td>22</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>The analyzed raw data for spectrophotometer reading of four of <em>M. lyrata</em> from Buntal.</td>
<td>32</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Genetic distance of ITS1 region across four individual from species <em>M. lyrata</em> and one sequence of species <em>M. lusoria</em></td>
<td>50</td>
</tr>
</tbody>
</table>
## List of Figure

<table>
<thead>
<tr>
<th>Figure No</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1</td>
<td>Schematic diagram of the nuclear ribosomal DNA internal transcribed spacers in eukarytes</td>
<td>12</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Schematic diagram of the nuclear ribosomal DNA internal transcribed spacers in eukaryotes The arrow indicated the region of ITS that cut by primer ETTS1 and ETTS2</td>
<td>20</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Features of <em>M. lyrata</em> shell morphology</td>
<td>26</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Left lateral view of exterior of left valve.</td>
<td>26</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Right lateral view of interior of left valve.</td>
<td>27</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Dorsal view of articulated valves.</td>
<td>27</td>
</tr>
<tr>
<td>Figure 4.5a</td>
<td>Gel electrophoresis photograph showing genomic DNA extraction product from the tissues samples of <em>M. lyrata</em> with phenol-chloroform protocol.</td>
<td>28</td>
</tr>
<tr>
<td>Figure 4.5b</td>
<td>Gel electrophoresis photograph showing genomic DNA extraction product from the tissues samples of <em>M. lyrata</em> with phenol-chloroform protocol.</td>
<td>29</td>
</tr>
<tr>
<td>Figure 4.6</td>
<td>Gel electrophoresis photograph showing PCR product of ITS region from six different individual of <em>M. lyrata</em>.</td>
<td>33</td>
</tr>
<tr>
<td>Figure 4.7</td>
<td>Electrophoresis analysis of the 16S rRNA PCR product obtained from four samples of <em>M. lyrata</em> using primer 16Sar-L and 16Sbr-H.</td>
<td>35</td>
</tr>
<tr>
<td>Figure 4.8</td>
<td>Gel electrophoresis photograph showing PCR product of COI region from six different individual of <em>M. lyrata</em>.</td>
<td>39</td>
</tr>
<tr>
<td>Figure 4.9</td>
<td>Example of gel electrophoresis photograph showing the purification of PCR product using Fermentas Purification Kit.</td>
<td>41</td>
</tr>
<tr>
<td>Figure 4.10a</td>
<td>Gel electrophoresis photograph showing PCR-RFLPs pattern using 6 restriction enzymes</td>
<td>43</td>
</tr>
<tr>
<td>Figure 4.10b</td>
<td>Graphic presentation of the gel photo</td>
<td>43</td>
</tr>
<tr>
<td>Figure 4.11</td>
<td>Sequence alignment of ITS region of four individual of <em>M. lyrata</em> with different species, <em>M. lusoria</em>.</td>
<td>48</td>
</tr>
<tr>
<td>Figure 4.12</td>
<td>A phylogenetic tree of <em>M. lyrata</em>, calculated by the neighbor-joining method</td>
<td>52</td>
</tr>
</tbody>
</table>
Preliminary Molecular Works on Hard Clam, *Meretrix lyrata* from Buntal Bay, Sarawak

Wong Fong Fun

Department of Aquatic Science  
Faculty of Resource Science and Technology  
University Malaysia Sarawak

ABSTRACT

*Meretrix lyrata* (lyrate Asiatic hard clam) could be found at Buntal Bay, Sarawak. This species is a filter feeder so it play important role in balancing aquatic ecosystem besides its important role as protein sources to coastal communities. Studies on *M. lyrata* in Sarawak had been limited to the morphological documentation and ecology. This study was designed to obtained baseline molecular data for *M. lyrata* from Buntal Bay, Sarawak. Standard molecular protocols were employed namely total genomic DNA extraction, Polymerase Chain Reaction (PCR), PCR product purification, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and direct sequencing. In addition, PCR product also subjected to PCR-RFLP. The 16S rRNA (approximately 650bp) and ITS region (approximately 1400bp) had been successfully amplified from *M. lyrata*. PCR-RFLP profile had also been documented by digestion of ITS PCR product with six different enzymes namely *Csp* 6I, *Hind* III, *Taq* I, *Mbo* II, *BsuR* I and *Mn* II. Based on ITS sequences, the genetic distance between individual found in Buntal area ranged between 0% and 0.3%. Low genetic distance was observed between *M. lyrata* from Buntal and China which ranged between 0.3% and 0.7%.

Keyword: *M. lyrata*, total genomic DNA extraction, 16S rRNA, ITS region, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

ABSTRAK


Kata kunci: *M. lyrata*, pengekstrakan total genomic DNA, 16S rRNA, ITS gen, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)
1.0 Introduction

*Meretrix lyrata* is a species from the genus *Meretrix* of the family Veneridae, or commonly known as lyrate Asiatic hard clam. It belongs to the eurythermal and euryhaline benthic bivalves in intertidal zone and it is very important marine aquatic economic animals (Lin, 2008). *Meretrix sp.* is a nutritious and delicious food having a specific flavor. It can be eaten boiled, steamed or roasted. *M. lyrata* has become common food consumed by the inhabitants of many parts of the island of Borneo.

Different habitat types and geographical isolation of clams for a long time have caused by long curved coastline and intricate marine geographic forms (Lin, 2008). Therefore, *M. lyrata* have demonstrated diversities in terms of shell shape, color and pattern in furthermore. Their genetic bases can be used to determine these consistent variances among population. There are still many controversies about the classification of the genus *Meretrix* in academy. Phenotypic traits and quantitative genetic methods on the molecular level, and molecular marker analysis technique can be use to investigate the genetic bases and their variances of representative populations of *Meretrix* clam (Lin, 2008).

Based on studied done (Canapa et al., 2003; Cheng et al., Kapnner & Bieler, 2006; Insua et al., 2003), the mitochondrial large ribosomal subunit 16S and Nuclear ribosomal first internal spacer region (ITS1) had shown remarkable success in constructing the phylogenetic structure of the species and reveal the evolution relationship upon wide variety of Bivalve. In Bivalve mollusks, the ITS region or any of two spacers has been studied in several species. The
available information comes from PCR amplification alone or PCR amplification followed by restriction analysis or sequencing, has been used to differentiate related species and to explore the phylogenetic relationship among bivalve species. A study of the phylogenesis of Veneridae was carried out using a partial sequence of the mitochondrial 16S mtDNA gene, which would definitely contribute to the understanding of the species belonging to this family (Cheng et al., 2006).

The purposes of this study are to establish molecular biology protocols (DNA extraction, PCR, Purification and PCR-RFLPs) that can be employed on *M. lyrata*. Besides that, it is also means to explore appropriate protocol to obtain good quality of total genomic DNA from *M. lyrata*. Then the 16S mtDNA gene and ITS region from *M. lyrata* will be amplified followed by sequence. The PCR product will then be subjected to Restriction Fragments Length Polymorphism to document *M. lyrata* restriction profile. The sequence will be analyzed to determine the genetic distances among sampled *M. lyrata* population.

Most of the studies on *M. lyrata* in Malaysia were carried out on their diversity, distribution and feeding behaviors. A quick search on NCBI database revealed that there is no genetic information on *Meretrix sp.* from Malaysia. This study is designed to employ molecular biology techniques on the two species in order to determine their genetic information. This genetic information will be useful in future research such as phylogeny and phylogeography of hard clams.
2.0 Literature Review

2.1 Morphology, Ecology and Importance

The classification of \textit{M. lyrata} according to Global Biodiversity Information Facility is as such:

Phylum: Mollusca

Class: Bivalvia

Order: Veneroida;

Superfamily: Veneroidea

Family: Veneridae

Genus: \textit{Meretrix}

Species: \textit{Meretrix lyrata}

\textit{M. lyrata} is characterized by its thick shell which moderately inflated with a variable, inequilateral shape, subtrigonal in outline (Ministry of Agriculture and Rural Development, n.d.). Their shells are triangle in shape and rough with growth ring emerge on the shell surface of the anterior part. However their internal margins smooth without any growth rings. The hinge plate is moderate in strength and shows no attrition. Each valve has three radiating cardinal teeth. They have light yellow or milky white color on the exterior of the shell, however some are found in brown veins. Their interior of shell is white in color. Their shell can achieve a maximum length of 6 cm (Ministry of Agriculture and rural development, n.d.).
*M. lyrata* distributed along the coastal area of Tropical West Pacific, from western Indonesia to the Philippines; north to the East China Sea and Taiwan Province of China, and south to southern Indonesia (Ministry of Agriculture and Rural Development, n.d.).

*M. lyrata* is a filter feeder and mainly consume on organic detritus and phytoplankton. Its growth rate varies seasonally with fast growth in May and June and slow growth in October and May. The reproduction season of hard clam starts from March to June and sometime to October. Their exploitation seasons are from February to May (Ministry of Agriculture and Rural Development, n.d.).

*M. lyrata* burrow themselves into sandy habitats and mud bottoms to protect themselves from wave impact, temperature fluctuations, desiccation and predation. They inhabit in intertidal and shallow sublittoral waters to a depth of about 20 m. their salinity toleration varies between 7 and 25\(^{0}/\text{oo}\) (Food and Agriculture Organization of the United Nations [FAOSTAT], n.d.).

Due to filter feeding, bivalves can accumulate xenobiotic compounds, therefore are organisms of choice for environmental monitoring, but also constitute potential risks for human health when they are consumed (Saavedra & Bachere, 2006). The meat of hard clam is delicious and processed into specialties. Hard clam is an important export item of the coastal provinces in eastern south of Vietnam. They can be eaten fresh, and processed under steamed, boiled and roasted forms. In Vietnam and the Gulf of Thailand, local people usually collected them for nutrient rich and economic food. They are commercially important in the Malacca Straits area (Indonesia) and appear in local markets of the southern Philippines (FAOSTAT, n.d.).
2.3 Total Genomic DNA Extraction

DNA-base techniques used for detecting genetic divergence within and among population such as PCR amplification of polymorphic genetic markers are highly contribute to recent phylogenetic studies (Aranishi & Okimoto, 2006). The ability to prepare acceptable quality and quantity genomic DNA sometimes becomes the limiting factor in determining the feasibility of the DNA-base techniques however (Aranishi & Okimoto, 2006).

Mikhailova and Johannesson (1998) had compare three different procedures for extracting high molecular weight genomic DNA of *Littorina*; phenol-chloroform, hexadecyltrimethyl ammonium-bromide (CTAB) and Chelex 100. In their research, they claim that the high amounts of high quantity DNA can be extracted by using both double phenol-chloroform and CTAB extractions. However Chelex 100 gave less amount of DNA. They had claimed that with double extraction, the DNA yield was in higher quality and was more effective for DNA amplification compare with single extraction. Large quantity of high molecular weight DNA without heavy contamination of degraded DNA had been extracted using Phenol chloroform extraction. The extraction using the CTAB buffer mostly yield high molecular weight DNA and some RNA. Besides that, they had found that the extraction method which yield high amount of DNA does not always give better DNA amplification product compare with the extraction method which give less DNA. The Chelex method gave a better result after amplification compare with CTAB extraction which gave higher concentration of DNA.
The polymerase chain reaction (PCR) is the repetitive bidirectional DNA synthesis via primer extension of a region in nucleic acid (Dieffenbach & Dveksler, 1995). To perform DNA synthesis two oligonucleotide primers, the four deoxynucleotide triphosphates (dNTPs), magnesium ions in molar excess of the dNTP, and a thermostable DNA polymerase are required in PCR amplification. For each application, the quantities of oligonucleotide primers, dNTPs, and magnesium may vary for each application (Dieffenbach & Dveksler, 1995).

Three distinct events must occur during a PCR cycle, firstly is the denaturation of the template then followed by primer annealing, and DNA synthesis by a thermostable polymerase. DNA denaturation occurs when the reaction is heated to $92^\circ\text{C-96}^\circ\text{C}$ to stop all enzymatic reactions. The time required to denature the DNA depends on its complexity, the geometry of the tube, the thermal cycler, and the volume of the reaction. The addition of glycerol, longer denaturation times, and the use of nucleotide analogs are required for DNA sequences that have a high G+C content to improve the yield of the PCR (Dieffenbach & Dveksler, 1995). High molecular weight genomic DNA may fail to completely dissociate if it is too low a temperature or too short a denaturation phase. However excessive denaturation will reduce enzyme activity (Palumbi et al., 1991).

After denaturation, the temperature is lowered so that the oligonucleotide primers can bind to their complementary single-stranded target sequences. The temperature of this step varies from $37^\circ\text{C}$ to $65^\circ\text{C}$, depending on the homology of the primers for the target sequence as well as the
base composition of the oligonucleotides. Primers are shorter in length and present at a significantly greater concentration than the target DNA, as a result, they hybridize to their complementary sequences at an annealing rate several orders of magnitude faster than the target DNA duplex can reanneal (Dieffenbach & Dveksler, 1995).

The last phase allows the extension of the oligonucleotide primer by a thermostable polymerase. Taq polymerase worked well at 72°C then this portion of the cycle is carried out at 72°C. The time required to copy the template fully depends on the length of the PCR product.

Each PCR is likely to require specific optimization for the template/primer pairs chosen owing to there is no single set of conditions that is optimal for all PCR. Many problems can be result from lack of optimization, such as no detectable PCR product or low efficiency amplification of the chosen template; the presence of non specific bands or smeary background; the formation of “primer-dimers” that compete with the chosen template/primer set for amplification; or mutations caused by errors in nucleotide incorporation. Since there are various parameters involved, optimization of a particular PCR can be time consuming and complicated. The parameters that had been determined are quality and concentration of DNA template, design and concentration of primers, concentration of magnesium ions, concentration of the four deoxynucleotides (dNTPs), PCR thermal cycling conditions, PCR buffer systems, selection and concentration of DNA polymerase, addition and concentrations of PCR cosolvents; and use of the “Hot start” technique. Optimization of PCR may be affected
by each of these parameters individually, as well as the combined interdependent effects of any of these parameters (Dieffenbach & Dveksler, 1995).

### 2.5 Gene of Interest

#### 2.5.1 ITS Region

In the nuclei of eukaryotic cells, the rDNA usually exists as tandemly repeated elements, each repeat unit composed of a transcription unit and a non-transcription unit which also called an intergenic spacer. The transcription unit includes an external transcribed spacer (5’ETS) followed by the 18S rRNA gene, ITS-1, the 5.8S rRNA gene, ITS-2, and the 28S rRNA gene. The genes are usually more highly conserved than the nontranscribed region.

Ribosomal DNA is particularly useful for phylogenetic analysis since it has both rapidly and slowly evolving regions. For comparing distantly related species, the slowly evolve coding region are used in while in comparing more closely related groups, the more rapidly evolving external transcribed spacer (ETS) and internal transcribed (ITS) regions are used in (Fernandez et al., 2001). ITS sequences are routinely used to distinguish related species and to infer phylogenetic relationships from populations to families and even higher taxonomic levels since ITS sequences show more divergence than their flanking regions and easily amplified (Cheng et al., 2006).
In the field of mollusk species identification, a variety of methods, PCR amplification alone or PCR amplification followed by restriction analysis or sequencing, has been used to differentiate related species and to explore the phylogenetic relationship among bivalve species. The ITS region or any of the two spacers has been studied in several species. PCR-RFLP of nuclear DNA fragments has been useful in distinguishing Scallop species (Lopez-Pinon et al., 2002), ITS-1 RFLPs to discriminate several mud crab species (Imai et al., 2004), sequence analysis of rDNA Internal transcribed spacer region in molecular phylogeny of Heterodonta (Canapa et al., 2001) and some scallop species (Insua et al., 2003).

Insua et al., (2003) had amplified and sequenced the internal transcribed spacer (ITS) region of the ribosomal DNA from the European scallops *Aequipecten opercularis*, *Mimachlamys varia*, *Hinnites distortus*, and *Pecten maximus*. The size of ITS reported ranged from 636 bp and 713 bp and GC content ranged between 47% and 50% among these species. Tree with similar topology always produced in despite of using ITS1 and ITS2, or both spacer sequences. Their results provide new insight into the evolutionary relationship of the scallop species and corroborate the close evolutionary relationship between the tribes Aequipectinini and Pectinini previously deduced from 18S rDNA. Minimal Intra-individual variation of the ITS sequence had been detected in *M. varia* and *P. maximus*. It can be say that intra-individual variation is more moderate compare with other animal species if the variation does exist. Insua *et al.* (2003) had claimed that both ITS1 and ITS2 exhibit extensive sequence variation among scallops. They found that the indels occur frequently throughout the sequence, but the repeated elements were seldom among scallops ITSs. This suggested that the nucleotide substitution may obscure the repeated elements if existed.
PCR-RFLP analysis of the internal Transcribed Spacer (ITS) Region for identification of 3 clam species (*R. decussates*, *V. pullastra*, and *R. philippinarum*) had been conducted by Fernandez *et al.*, (2001). PCR amplification using primer base on based on nucleotide sequences of *Mytilus* ITS regions produced a different fragment length in three clam species. Research by Fernandez *et al.*, (2001) had claim that specific restriction profiles yielded by digestion of the PCR products with endonucleases *HinF* I and *Rsa* I, followed by agarose gel electrophoresis of the digested products had enabled direct visual identification of the species analyzed.

Remigio and Blair (1997) used the ITS1 and ITS2 of the nuclear ribosomal DNA internal transcribed spacer sequences to reinvestigate the relationship among four problematic North American stagnicoline snails species. Subgenus *Stagnicola* s.str from the northen U.S.A represented by three species; *Stagnicola catascopium*, *Stagnicola emarginata* and *Stagnicola elodes* whereas the subgenus *Hinkleyia* represented by closely related Canadian species (*Stagnicola caperata*). In their research, they found that three members of the subgenus *Stagnicola* s.str had very similar ITS sequence. This indicated that they were very similar and such data could be taken as evidence that the snails are conspecific or are sister species. Besides that, they have shown the most useful markers in ITS gene to distinguished two very closely related subgenera. They had state that the nuclear rRNA genes occur in long clusters of tandem repeat this will likely induced intra-individual or interindividual sequence variation.

Cheng *et al.*, (2006) had carry out a study on sequences of ribosomal DNA Internal Transcribed Spacers of clams, which would definitely contribute to the understanding of the
species belonging to the family Veneridae. The first and second ITS regions of the rDNA from four species, *M. meretrix*, *Cyclina sinensis G*, *Mercenaria mercenaria* and *Protothaca jedoensis L.*, belonging to the family Veneridae were amplified by PCR and sequenced. They stated that the size of the ITS1 sequence ranged from 585 bp to 900 bp is the largest range reported thus far in bivalve species. Their study had showed that *M. meretrix* had the longest ITS1 sequence (900 bp) with CG content of 61.67% and ITS2 sequence (412 bp) with CG content of 65.29%. They claim that the size of ITS varies between species. Both ITS1 and ITS2 regions in family Veneridae were exhibited extensive sequence variation and obvious length polymorphisms. The study showed that ITS1 regions had weak phylogenetic discrimination power in Veneridae since it had high length variation, large number of indels and the presence of tandem repeated sequences. ITS2 were more effective in the construction of topology in Veneridae clam since the length of ITS2 was shorter than of ITS1. ITS2 sequence was therefore an efficient tool in revealed an evolutionary relationship among these organisms and applied in establishing species relationship.

![Schematic diagram of the nuclear ribosomal DNA internal transcribed spacers in eukaryotes](Adopted from Cheng et al. 2006)

2.5.2 **Mitochondrial Large Ribosomal Subunit (16S)**

Phylogenetic history across a very broad spectrum had been inferred through studies of rDNA sequences. The rRNA was systematic versatile because of presence of many copies of most rDNA sequences per genome, and the pattern of concerted evolution that occurs among
repeated copies. In addition, rRNA was very useful for constructing “universal” primers due to
the island of highly conserved sequences within most rRNA and for amplifying regions of
interest by use of the Polymerase Reaction Chain or for use of probes in restriction enzyme
analysis. 16 rRNA is one of the most studied small subunit nuclear gene because of it is
among the slowest evolving sequences found throughout living organism. The slow rate of
changes permits the construction of many nearly universal primers, which facilitates
sequencing effort from groups that had not been studied previously (Hillis & Dixon, 1991).

The study on molecular data from the 16S rRNA gene for the phylogeny of Veneridae had
been carried out by Canapa et al., 2003. This study focused on the clarification of the
systematic relationship between the species in the family Veneridae. The comparison was
done prior to the analysis of the portion of the nucleotide sequence of the gene encoding the
large subunit of mitochondrial ribosomal RNA for 14 species representative of 10 genera
belonging to the 6 different subfamilies. The 16S rDNA sequences obtained in this study range
from 462 bp (Globivenus effossa and Mercenaria mercenaria) to 596 bp (Meretrix lyrata)
nucleotides in length (excluding the primers) in group, whereas the outgroup sequences are
470 and 483 nucleotides long in Ensis ensis and Spisula subtruncata, respectively. From the
result obtained using the maximum- parsimony and neighbor-joining methods indicate that the
natural subdivision was not always correlation with the current placement of the genera into
subfamilies.
2.6 PCR-Restriction Fragment Length Polymerase

With the advent PCR, short region of DNA surrounding the restriction site of interest is possible to amplify followed by restriction digestion of the product from genomic DNA (Newton & Graham, 1994). According to Hillis et al., (1996), restriction endonucleases (REs) are enzymes isolates from bacteria that cut DNA at a constant position within a specific recognition sequence, typically four to six base pair long. REs can be used to detect the base substitutions or insertion event in genomic DNA. Characteristic recognition site in DNA cleave by each restriction endonucleases is usually symmetrical and leaves ends with a 5’ overhang, a 3’ overhang, or no overhang when cleaved. The specificity of cleavage by REs means that complete digestion of a particular DNA allele will yield a reproducible array of fragment.

Particular DNA allele that digested with restriction enzymes revealed variation in fragment pattern referred to Restriction fragment length polymorphisms (RFLP). Base substitutions can create or eliminate cleavage sites for a particular enzyme, thereby altering the number and size of fragment detected by that enzyme alone.

In the field of mollusk species identification, PCR-RFLP of nuclear and ribosomal DNA and mtDNA fragments has been useful in distinguishing species of zebra mussels (Baldwin and others 1996), clams (Fernandez et al., 2001), and oyster (Boundry et al., 1997). PCR-RFLP of mitochondrial DNA have been used in the research of Boudry et al., (1997) to examine
genetic differentiation between populations of the Portuguese oyster, *Crassostrea angulata*, and the Pacific Oyster, *Crassostrea gigas*.

Research by Baldwin *et al.*, (1996) suggest that Restriction fragment length polymorphisms (RFLPs) of the mitochondrial cytochrome oxidase subunit gene (COI) could provide rapid and relatively inexpensive diagnostic markers for identify molecular genetic differences is useful for identification and quantification of larvae from five species. Potential restriction-sites that could putatively produce species-specific fragments had identified by searching the COI sequences from the five species. Digestion of COI with *Nla*I V had produced diagnostic fragment profiles for each of the six target taxa with different fragment sizes each of them.

Lopez-Pinon *et al.*, (2002), using the polymerase chain reaction and restriction fragment length polymorphism (PCR- RFLP) of the ribosomal DNA region spanning the 5.8S RNA gene and the two flanking internal transcribed spacers (ITSs), DNA-based molecular markers for the identification of the scallops *Aequipecten opercularis*, *Chlamys distorta*, *Mimachlamys varia* and *Pecten maximus* had established. Digestion of the PCR product with endonucleases *Alu* I, and *Sma* I yielded specific restriction profile in *A. opercularis*. In their research, only one individual of *A. opercularis* out of a sample of 70 showed evidence of intraindividual polymorphism for the restriction site of *Sma* I, Therefore, it seems that in the scallop studied, the concerted evolution of the ITS is strong enough to maintain sequence uniformity o at least very low levels of intraspecific polymorphism.
3.0 Materials and Methods

3.1 Field Sampling

Samples used in this study were collected in the coastal of Buntal. Global Position System (GPS) was used to record the location of sampling site. Collection of the hard clams was carried out during the spring tide cycles and the collectors had to wait until low tide. Hard clam were put in seawater so that it can stay alive for few hours. All calms were transported back to UNIMAS Aquatic Molecular Lab. After that, the hard clams were washed with tap water and further stored at -80°C.

3.2 Morphology Assessment

The photographs of hard clam’s morphology was captured and observed. Identification was carried out using taxonomic keys provided by Mikkelsen (2006).

3.3 Total genomic DNA extraction

3.3.1 CTAB-chloroform DNA extraction

CTAB-chloroform DNA extraction was performed in this process (Doyle and Doyle, 1987). In order to make the foot tissue easier to be minced, the samples of clams were dipped into the 90% ethanol before the mincing procedure beginning. To extract total genomic DNA, 700 µl of 2 X