Preliminary Morphological and Molecular Study on Ghost Crabs from Satang Island and Sematan, Sarawak

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DECLARATION

I hereby declare that no portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualifications of this or any other university or institution of higher learning.

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List of Abbreviation

µL	micro liter
rpm	round per minutes
bp	base pair
EtOH	Ethanol
NaCl	Sodium Chloride
ddH_2O	Double Distilled Water
$MgCl_2$	Magnesium Chloride
CTAB	Cetyl Trimethyl Ammonium Bromide
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
EtBr	Ethidium Bromide

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ABSTRACT

Ocypode ceratophthalmus commonly known as ghost crabs could be found abundance around the sandy beaches area worldwide. They act as bio-indicator for clean sandy beaches as well as pest to turtles conservation sites. Several studies related to *O.ceratophthalmus* on ecology, behaviour and morphology had been carried out outside Malaysia but no study had been carried out in Sarawak beaches. Therefore, this study is designed to document the morphological characteristics of the ghost crabs and to access the genetic diversity among ghost crabs from Satang Island and Sematan area. Modified CTAB protocol was used to obtain the total genomic DNA , followed by PCR-RAPD technique using primer OPA-01. In addition, weight, carapace length, carapace width, chelipeds height and chelipeds length were recorded. PCR-RAPD profile using OPA-01 primer was successfully generated 86.36% polymorphism. For male ghost crabs, there was no significant different (T-test, $p>0.05$) between Satang Island and Sematan sample in term of carapace width, carapace length, chelipeds height, chelipeds width and weight. For female ghost crabs, there only have significant different (T-test, $p>0.05$) between Satang Island and Sematan sample in term of carapace length and carapace width while there is no significant different (T-test, $p<0.05$) in terms of chelipeds height, chelipeds length and weight.

Keywords: *Ocypode ceratophthalmus*, ghost crabs, PCR-RAPD, OPA-01, morphology

ABSTRAK

Ocypode ceratophthalmus dikenali sebagai ketam hantu dan banyak dijumpai di sekitar pantai berpasir seluruh dunia. Mereka bertindak sebagai penunjuk biologi untuk pantai dan menjadi pemangsa di laman pemuliharaan penyu. Beberapa kajian berkaitan dengan O.ceratophthalmus pada ekologi, perilaku dan morfologi telah dilakukan di luar Malaysia namun belum ada kajian yang dilakukan di pantai Sarawak. Oleh kerana itu, kajian ini direka untuk mendokumentasikan ciri-ciri morfologi ketam hantu dan mengakses kepelbagaian genetik antara ketam hantu dari Pulau Satang dan kawasan Sematan. Kaedah CTAB DNA yang telah diubahsuai digunakan untuk mendapatkan produk DNA. PCR-RAPD telah dijalankan dengan menggunakan primer khusus yang dikenali sebagai OPA-01. Selain itu, berat, panjang dan lebar karapes serta tinggi dan panjang chelipeds telah direkodkan. PCR-RAPD profil menggunakan primer OPA-01 telah berjaya menghasilkan 86.36% polimorfisme. Tiada perbezaan terdapat pada ketam hantu jantan, (T-test, $p> 0.05$) antara Pulau Satang dan Sematan dalam lebar karapes, panjang karapes, ketinggian chelipeds, lebar chelipeds dan beratnya. Untuk ketam hantu betina, tiada perbezaan yang ketara (T-test, $p> 0.05$) dapat dilihat antara Pulau Satang dan Sematan pada ketinggian chelipeds, panjang chelipeds dan berat sementara terdapat perbezaan yang ketara (T-test, $p <0.05$) pada panjang dan lebar karapes.

Kata kunci: Ocypode ceratophthalmus, ketam hantu, PCR-RAPD, OPA-01, morfologi

1.0 INTRODUCTION

Crabs belong to a group of animals known as decapods crustaceans (Oriola *et al*., 2005). *Ocypode ceratophthalmus* that is commonly known as horned eye ghost crabs are the most widespread organism and it can be found in many parts of the world (Ruppert and Barnes, 1994; Barros, 2000). Ghost crabs are semi-terrestrial crabs and typically populated at tropical and subtropical sandy beaches area (Dahl, 1953; Hedgepeth, 1957; Barras, 1963; Jones, 1972; Wolcott, 1978).

Crabs body are divided into head, thorax and abdomen. They have chitinous exoskeleton and each segment of their body bears and jointed with appendages. Crab body cavity filled with the haemolymph or known as haemocoel and they breathe through their gills (Ruppert and Barnes, 1994). Ghost crabs have stalked eyes and 5 pairs of walking legs (Ruppert and Barnes, 1994; Dhamrah *et al*., 1992). Ghost crabs are nocturnal organisms which they emerge from their burrows at dusk and start their activity such as go down to the water edge to prey on clams, mole crabs and sea turtle hatchlings or to scavenge for food and digging shallow holes in the sand below the drift line (Wolcott, 1978; Ruppert and Barnes, 1994; Strachan *et al*., 1999). Ghost crabs are important as an organic carbon cycles since they eat the detritus and dead organisms at the beach area. They are also important as bio-indicators and become valid tools in the rapid identification of environmental impact (Barros, 2000; Neves, 2006).

Random Amplified Polymorphic DNA techniques or RAPD are known for its utility in carrying out the initial screenings in many loci. Besides that, RAPD technique also is used to distinguish different species of organism (Lynch and Milligan, 1994). PCR-RAPD marker is a simple technique an inexpensive (Bardacki, 2001) because it only

require the low quantities and medium quality of total genomic DNA to conduct (Amavet *et al*., 2007).

A lot of study about the ghost crabs have been conducted by Haley (1973), Wolcott (1978), Vries (1994), Weinstein (1995), Strachan *et al*. (1999), Ttott (1999) and Moss and Mcphee (2006) but all of the study were carried out at the other countries. Very few studies on ghost crabs in Malaysia especially in Sarawak beaches were carried out. Therefore this study is designed to obtain the baseline data for ghost crabs from Satang Island and Sematan sandy beaches area.

The objectives of this study include:

1. To record the PCR-RAPD profile of ghost crab from Satang Island and Sematan sandy beaches.
2. To document morphological and morphometric data characters of ghost crabs from Satang Island and Sematan sandy beaches.

2.0 LITERATURE REVIEW

2.1 Ghost Crabs

According to Ruppert and Barnes (1994) and Barros (2000), ghost crabs from genus *Ocypode* are the most widespread organism from Ocypodidae Family and it can be found worldwide. According to Weinstein (1994), ghost crabs was known because of their fast speed while moving and also because they are nocturnal organism which they went out for prey in night time.

2.1.1 Behaviour

According to Weinstein (1994) ghost crabs can moves continuously over long distances at the high speed. Ghost crabs are nocturnal organisms (Weinstein, 1994 and Strachan *et al*., 1999) where they have tendency to emerge from their burrows starting from dusk and they are more active during night compared to daytime. Upon ghost crabs emerging from their burrows at dusk, they will go directly towards the water body before they begin their nocturnal activity such as digging shallow holes in the damp sand below the drift line (Wolcott, 1978). He also stated that ghost crabs will stop their digging activity on the foreshore at dawn and then ghost crabs will begin to move towards inland to search for suitable burrows for daytime.

Strachan *et al*. (1999) reported that the burrow depth of the ghost crabs usually increase as their burrow far away from the water body. This is because the burrows of the ghost crabs needs to be located at least 1cm above the water table in order to enable them to do respiration by using their fine hair which located near in their walking legs. Juveniles crabs or small crabs have higher rates of water loss compared to the larger individuals

(Eshky, 1985), therefore, most of the smaller crabs burrows could be found in the zone that are close to the sea or water body (Strachan *et al*., 1999; Naves *et al.,* 2006). Besides that, smaller ghost crabs also can be found wondering around the sandy beach area during day time. Strachan *et al*. (1999) claimed that the smaller crabs do not have the ability to make their own burrows yet therefore, they use other crabs burrows when it is available.

2.1.2 Ecology

Wolcott (1978) carried out a study on ecological role of ghost crabs, *O. quadrata* on an ocean beach. He disagree with other researchers that ghost crabs are not primarily a scavenger (Cowles, 1908; Cott, 1930; Phillips, 1940; Crane, 1941 and Hedgepeth, 1957). Many studies (Cowles, 1908; Cott, 1930; Phillips, 1940; Crane, 1941; Hedgepeth, 1957), stated that ghost crabs are primarily scavengers to the other organism.

Ghost crabs seldom appear to feed at the higher part or at the upper beach (Wolcott, 1978). Usually, before the ghost crabs eat, they will quickly approach to investigate the objects first and then they will occasionally feed upon the large objects such as barnacles or dead organism which were washed away by the sea water (Wolcott, 1978). Ghost crabs are predators under the normal circumstances and their diet usually consists of over 90% live prey such mole crabs, clams, hatchling sea turtles, lizards, land crab, hermit crab, fiddler crab and insects (Wolcott, 1978; Strachan *et al*., 1999). Hughes (1966) stated that *O. ceratophthalmus* will plays an important role as predators and when their population is higher or abundance and sometimes they could also can become scavengers.

2.1.3 Importance

Ghost crabs are important in term of ecology in the environments. However, there is no study on the importance of ghost crabs in terms of of food value and socio-economic. Ghost crabs are important because they consume any organic materials such as organic detritus and live animals from their environments and transfer the energy from one trophic level to the higher trophic levels in food web (Wolcott, 1978; Wolcott and Wolcott, 1984).

Anthropic impacts in coastal areas have been increasing intensely and give an adverse effect on the flora and fauna environments (Neves *et al.*, 2006). Urban development on the sandy beaches area also have caused most of the dunes to be remove to build roads and building (Ranwell and Boars, 1986). Besides that, leisure activities also had a potential to give impact on environments. Few studies related on ghost crabs as potential indicators in sandy beaches area had been carried out (Defeo and De Alava, 1995; Jaramillo *et al.*, 1996; Barros, 2001; Neves, 2006).

Worren (1990) had carried out a study and used the burrows of the ghost crabs in sandy beaches area to determine the populations in sandy beaches area. This study was successes and have proved that there present a strong correlations between the numbers of burrows of the ghost crabs with its number.

Neves *et al.* (2006) had carried out the study on *O. quadrata* as a potential indicator of anthropic impacts in Brazil sandy beaches area. In this study, the samples of ghost crabs were taken at three different areas which have different level of anthropic impacts. This study successfully showed that the density of ghost crabs burrows can be used as rapid tool in determining the anthropic impacts in coastal zone area.

2.1.4 *Ocypode ceratophthalmus*

Taxonomy of *O. ceratophthalmus* or commonly known as Horned eye ghost crab is shown in Table 2.1.

Kingdom	: Animalia
Phylum	: Arthropoda
Subphylum	: Crustacea
Class	: Malacostraca
Order	: Decapoda
Family	: Ocypodidae
Genus	: *Ocypode*
Species	:*Ocypode ceratophthalmus*
Common name	: Horned Eye Ghost Crab

Table 2.1: Taxonomy of Horned Eye Ghost Crab, *Ocypode ceratophthalmus*. (Ruppert and Barnes, 1994).

Wolcott (1978) and Ruppert and Fox (1988) state that ghost crabs use gills for their respiration, they will return occasionally to the water to wet their gills. Besides returning to water, they also can moisten their gills by extracting water from damp sand by using the fine hairs near the base of their walking legs to wick ground water up to the gills through their capillary action (Ruppert and Fox, 1988). Burrows of the ghost crabs are mostly can be found at the high marks on the upper beach and generally the number of the burrows

will decrease with decreasing height of the shore (Ruppert and Barnes, 1994; Strachan *et al*., 1999; Barros, 2000). While burrowing, they will excavate the sand and form sand pile outside of their burrow opening. Ghost crabs ceased digging on the foreshore at dawn and they began to move inland to search for suitable burrow for daytime refuge (Wolcott, 1978; Strachan *et al*., 1999).

2.2 Molecular work (RAPD)

Random Amplified Polymorphic DNA techniques or known as RAPD is recognized for its ability in carrying out the initial screenings in many loci and it can distinguish many different species of organisms also can be distinguish simultaneously (Lynch and Milligan, 1994).

PCR-RAPD marker is a simple technique and it also does not require a lot of cost or inexpensive to conduct (Bardacki, 2001). Besides that, Amavet *et al*. (2007) also said that PCR-RAPD only require the low quantities and medium quality of DNA to detect the PCR-RAPD profile.

The RAPD technique is a PCR based technique and it allowes scores of markers to be assayed on DNA extracted from a single organism (Wilkerson *et al*., 1993). In conducting PCR-RAPD reactions, only a single of short primers used and usually the primer has ten bases in length of randomly sequence chosen. Williams *et al*. (1990) stated that the standard RAPD technology utilises short synthetic oligonucleotides of random sequences. Small amount of total genomic DNA was needed and low annealing temperature is required in carrying out PCR-RAPD reaction by using specific primer.

Wikerson *et al.* (1993) stated that the primer used in PCR-RAPD needs to match the binding site that approximately 2 to 3 kilobase pairs of another oppositely oriented binding site, therefore the single oligonucleotide can prime replication in both the forward and reverse direction and resulting the RAPD band. The number and size amplified fragments of PCR-RAPD depend on length and sequence of short, single and arbitrary primers (Bardacki and Skibinski, 1994). RAPD analysis also is multi-locus technique that detecting the polymorphism based on an amplification of random DNA segments using specific primer that have specific sequence (Amavet *et al.*, 2007).

Arnold *et al*. (1991) in their study stated that RAPD band may display a high degree of polymorphism, and multiple primers can be screened against taxa of interest. The study that had been carried out by Arnold *et al*. (1991) has proven to be a means of quickly in identifying species-specific markers.

The presence or the absence of the polymorphism in PCR-RAPD profile is either caused by nucleotide sequence divergence in primer sites or by insertions or deletions in the amplified segment of template DNA (Amavet *et al*., 2007). Williams *et al*. (1990) had mentioned that RAPD bands produced usually will represent the dominant genetic markers, which are inherited in a mendelian fashion or mendelian trait and it can be used as a molecular diagnostic characters at different taxonomic levels. This taxonomic identification and population genetic surveys have been successfully applied by Hadrys *et al*. (1992) in their study.

Neto *et al.* (2007) had carried out a study on population of the mangrove crabs *Ucides cordatus* to see the genetic variables of the crabs through the wide area. In this study, PCR-RAPD approach was used to see the differences of the genetic mapping. As a result, they found out that mangrove crabs in the wide range area around the estuaries shared the same pattern of genetic variation.

Wang *et al*. (n.d) use PCR-RAPD approached in their study to see the differences in cactus plants because of the difficulties to distinguish this species from field observation due to small differences in characteristics found in the plant species. They had successfully determine the different species of the cactus plant. Bardacki (2001) had successfully used PCR-RAPD to determine different types of species and sub-species in tilapia. Besides that, PCR-RAPD also was used in detecting or diagnoses a disease. A study on hydatid cysts *Echinococcus granulosus* was conducted by Al-Fayadh *et al*. (2010) in which their aim is

to see how different patient from various age gap was affected by this disease. In addition, they also tested it to the different animals such as cow using different tissues liver, lungs, ovary and spleen. PCR-RAPD approached had been successful in determining or diagnosis the disease.

2.3 Morphological Studies

Morphometric data of the crabs on relative growth is often used to determine any changes in form and size of the abdomen, pleopods or chelipeds during ontogeny (Fumis *et al.,* 2000). Hartnoll (1974) stated that relative growth will occur as the animals growth progress. Relative growth is defined as dimension of certain part in animals body that will grow much more than the other part in the animals body as the growth of the animals progress (Hartnoll, 1974). Rodrigues (1985) stated that relative growth is a morphometric relationship that use mathematical equation to describe and related the dimension of body parts or organs with the entire body. For crustacean populations, the size at sexual maturity is an important aspect in the life history of the species (Stearns and Koella, 1986). Sexual maturity is the set of morphological and physiological transformations where young or immature individuals gain the ability to produce gametes for reproduction. For morphological studies, Abellan *et al.* (2000) stated that crustacean will reach its morphometric maturity at the puberty moult which some changes in relative growth will occur in their appendages and the slope or the elevation of the line in graph also will change. In the other words, morphometric maturity is the crustaceans that have attained its maturity state and able to reproduce.

Abellan *et al*. (2000) had carried out a study on the morphometric, functional and sexual maturity of the red crab *Chaceon affinis* in Canary Island waters. The red crabs are important in fisheries sectors as the protein sources for surrounding people, therefore their study was conducted to collect data on the crabs so that proper management on the crabs harvesting could be implemented. In this study, they collected the crabs monthly by using variety of traps and the sample collection was done regularly for two years. They measured the morphometric characteristics of the crabs and the maturity state of the crabs were determined based on the data obtained.

Akin-Oriola *et al*. (2005) also carried out the study on the morphometric and meristic features of crustaceans in West America regardless of the importance of crabs as food values. This study also was conducted to provide the information that needed for the effective management and utilization of this resource. From this study, two different types of crabs were measured and the observation on the growth and factors that affecting both of the crabs growth was done. In this study, the sample of the crabs was collected every month to ensure that the data collected is significant.

Fumis *et al.* (2007) also had carried out research on morphometric study of the *Hexapanopeus schmitti* or known as xanthoid crab. In this study, allometric growth method was used to measure the growth pattern of the crabs in which for the samples were taken monthly for about two years in the Ubatuba region, northern coast of Sao Paulo, Brazil. The sample were collected using a fishing boat that was equipped with double-ring tow nets. The morphological characters such as carapace width, carapace length, female abdomen, propodus length, propodus height and gonadopods of male crabs were measured and recorded.

In 1973, a study that related to *Ocypode ceratophthalmus* or commonly known as horned eye ghost crabs was conducted by Haley (1973). The study that was carried out also about the morphometric measurements and the study were done in Hawaii. For this study, ghost crabs were collected regularly monthly for two years. In this study, the maturity of the ghost crab was done by observing the gonads and relate the maturity of the crabs with the morphometric data that they obtained.

3.0 MATERIALS AND METHODS

3.1 Sampling Sites

Figure 3.1 Sampling sites involved in this study; A is Sematan sandy beaches and B is Pulau Satang Besar

Samples of the ghost crab was collected along the sandy beach area of Sematan and Pulau Satang Besar (Figure 3.1). The sampling of the ghost crabs at Pulau Satang Besar (N01^{o} 46.828' E110 ^{o} 09.882') was conducted on 26 and 27 July 2010 while the sampling of the ghost crabs at Sematan (N01 ^{o} 49.416' E109 ^{o} 45.813') was conducted on 7 and 8 August 2010.

3.2 Sample Collection

Specimens of ghost crabs were collected by using custom made traps (Appendix A). The traps were placed randomly along the sandy beach area at Pulau Satang Besar and Sematan area. Around 15 to 20 individuals of ghost crabs were collected at each study area. The individuals of ghost crabs collected were stored and preserved in labelled plastic bottles that contained 70% Etoh whereas some of the live samples of ghost crabs were placed in the plastic container that was filled with sand. The samples were kept in surrounding temperature and were transported by bus to UNIMAS. Later, upon reach in the laboratory, the live samples of the ghost crabs were preserved in -80^{o}C freezer as a fresh samples for molecular work.

3.3 Laboratory Work

This section was divided into nine sections namely CTAB buffer preparation, totals genomic DNA Extraction, gel electrophoresis, gel documentation of DNA bands, optical density reading, PCR-RAPD, gel documentation of DNA bands for RAPD, morphometric measurement and statistical analysis.

3.3.1 CTAB Buffer Preparation

CTAB buffer is an important chemical to use in total genomic DNA extraction method. It helpes to lyses the cell membrane of tissue samples during the DNA extraction process. CTAB buffer was prepared with several mixture of chemical such as $NaCI_2$, EDTA, Tris-Base, CTAB, 2-mercaptoethanol-β- mercapto and ddH_2O (Table 3.1). $NaCl_2$, Tris-Base, CTAB and EDTA are chemicals in powder form. Later, those chemicals were mixed

together with ddH_2O in a beaker. Then, the beaker was placed on the hot plate with stirrer to ensure that the chemical in the solution mixed and dissolved properly. The mixed solution was placed on the bench to cool before it was transferred into a SCHOTT bottle. The SCHOTT bottle was covered with the aluminium foil before 2-mercaptoethanol-β-mercapto was added because 2-mercaptoethanol-β- mercapto is sensitive to light. The process of inserting 2-mercaptoethanol-β- mercapto was conducted in a vaporization chamber since it is hazardous. SCHOTT bottle that contain CTAB buffer was labelled properly with the name of the solution and the date of the preparation. Later, it was kept on the bench work in the lab at room temperature.

Table 3.1 Preparation of 500 mL of CTAB buffer

$NaCl_2$	40.90 g
Tris-Base	6.05 g
CTAB	100.00 g
EDTA	3.70 g
2-mercaptoethanol-β-mercapto	1000 µL
ddH_2O	500 mL

3.3.2 Total Genomic DNA Extraction

Fresh sample of ghost crabs were used for total genomic DNA extraction. A fragment of muscle tissues from the walking legs were removed and the genomic DNA was extracted by using the modified CTAB method from Doyle and Doyle (1987). An appropriate amount of 5 g of walking legs tissue samples was minced properly and several drops of CTAB Buffer was used to break the cell wall of the tissue. After the tissue was minced properly, the minced samples were placed into 2.0 mL microcentrifuge tube that contains 700 µL of 2× CTAB (Cetyl-trimethyl Ammonia Bromide) buffer (Grewe *et al*., 1993) and

5 µL of Proteinase K. Later, samples of minced tissue in the tube were incubated in the water bath at 60 ^{o}C for about 1 to 3 hours. Then, 700 µL of chloroform-isomyl alcohol was added into each of the tube and the tube was shake by using vortex for about 1-2 minutes to mix the solution properly. After that, the tubes were centrifuged using High-Speed Micro Centrifuge CFI5RX, at 13 000 rpm for 15 minutes in 4^{o}C temperature. The centrifuge process produced two layers in each microcentrifuge tubes. The upper layer aqueous phase was taken out slowly using micropipette and it was transferred into a new tube. Then, 500 µL of 100% EtOH was added into the tube as well and the tube was inversed slowly to make sure that the mixture is properly mixed. Then, it was left for about 30 minutes or it was incubate overnight before it is centrifuge again at 13 000 rpm for another 15 minutes at 4^{o}C. The excess EtOH was poured out and 500 µL of 70% EtOH was added into the tube. 25 µL of 3M $NaCl_2$ solution was added into the tube. The sample were centrifuged again at 13 000 rpm for 15 minutes at 4^{o}C. After that, the excess EtOH was poured and removed completely from the tube. Then, the tube that contained pellet was left at room temperature for drying. Finally, 50 µL of ddH_2O was added to the pellet to dissolve it and the sample was stored in the freezer at -20^{o}C.

3.3.3 Gel Electrophoresis

The DNA quality and approximate yield of total genomic DNA extraction were determined by electrophoresis in a 1% agarose gel containing EtBr at 90 V for 30 minutes. Agarose gel was prepared by weighing approximately 0.5 g of agarose powder using the analytical balance (AR3130 Adventures^{TM}, Ohaus Corporation) before it was add into the 250 mL beakers. After the agarose powder was added into the beaker, 50 mL of 1× TAE (Tris-acetate-EDTA) buffer was measured using 50 mL of measuring cylinder and later it also