Faculty of Resource Science and Technology

GENETIC DIVERSITY OF GREEN TURTLE (*Chelonia mydas*) FROM TALANG-TALANG ISLAND AND SATANG ISLAND, SARAWAK BASED ON COI GENE ANALYSIS

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Bachelor of Science with Honours

(Aquatic Resources Science and Management)

2011

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This project is submitted in partial fulfillment of the
Final Year Project (STF 3015) Course and requirement for the degree of
Bachelor of Science with Honours
(Aquatic Resource Science and Management)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2011

ACKNOWLEDGEMENT

First and foremost, I would like to thank god for giving me strength, constant spirit and the blessings that had kept me working continuously to ensure the success of this project.

Secondly, I would like to express my sincere gratitude and appreciation to my supervisor, Dr. Ruhana Hassan, for her kind help, dedicated guidance, encouragement and support that had assist and supervised me since the beginning of this project all the way to the completion of this project. I would like to extend my gratitude to my respective seniors Nurhartini Kamalia Yahya and Farah Adibah Isa, and also my labmates Nor Yasmin Kasim, Mohd Khairulazman, Uzma Hazwani Arif and Mohd Zulhelmi Zamri for their kind assistance, companionship and support that had helped me throughout this project.

Last but not least, all my love and greatest appreciation goes to my beloved father, Mr. Md Shahid Ismail and my mother, Mrs. Salmi Marzuki for continuous encouragement and support in every aspect. Thank you.

DECLARATION

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

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LIST OF ABBREVIATIONS

Abbreviation	Full Term
bp	Base pair
cm	Centimeter
CTAB	Cetyltrimethyl ammonium bromide
DNA	Deoxyribonucleic Acid
EDTA	EthyleneDiamine Tetra-Acetic Acid
g	Gram
L	Liter
µl	Microliter
ml	Mililiter
mm	Milimolar
mtDNA	Mitochondrial Deoxyribonucleic Acid
ng	Nanogram
nm	Nanometer
NCBI	National Centre for Biotechnology Information
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
rpm	Rotation per minute
UV	Ultraviolet

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ABSTRACT

Chelonia mydas (green turtle) is a long lived marine reptile with complex life histories that is extensively distributed across the globe and has become the quintessential subject for public conservation awareness. Many studies on C.*mydas* from Sarawak had been carried out in terms of population size, tagging experiment in assessing turtle migration and study on green turtle egg hatch rate. Several preliminary molecular works on C.*mydas* using D-Loop and 16S gene sequences had also been conducted. However, there is no study yet conducted on C.*mydas* from Sarawak, using Cytochrome Oxidase I (COI) gene sequence. Therefore, this study is aimed to sequence COI gene from C.*mydas* of Satang Island and Talang-Talang Island and the sequence data will be used to assess genetic variation between individuals and infer molecular phylogeny of C.*mydas*. This study involved the utilization of molecular work involving total genomic DNA extraction, Polymerase Chain Reaction, DNA sequencing and Phylogenetic Analysis. A total of 510bp of COI gene sequences were obtained from C.*mydas* in this study. Genetic divergence of 0% to 0.2% was recorded among C.*mydas* samples from both study sites indicating intraspecific variations between C.*mydas* samples. Phylogenetic trees constructed showed monophyletic of the C.*mydas* samples as they grouped together into a single clade.

Keyword: *Chelonia mydas*, COI, D-Loop, Genetic Divergence, Molecular Phylogeny

ABSTRAK

Chelonia mydas (penyu agar) adalah reptilia laut yang mempunyai jangka hayat yang panjang dengan kehidupan yang kompleks, mempunyai taburan populasi yang meluas di seluruh dunia dan telah menjadi perhatian subjek utama untuk pemuliharaan dan kesedaran awam. Beberapa kajian tentang penyu agar telah dijalankan di Sarawak seperti kajian tentang saiz populasi, eksperimen melalui 'tagging' untuk analisa migrasi dan juga kajian tentang kadar penetasan telur penyu. Beberapa kajian molekular juga telah dijalankan melibatkan gen liku-D dan 16S rRNA. Namun, tiada kajian tentang penyu agar di Sarawak menggunakan gen Cytochrome Oxidase I (COI). Kajian ini bertujuan untuk mendapatkan gen COI bagi sampel penyu agar di Pulau Satang dan Pulau Talang-Talang. Data kajian ini akan digunakan untuk menganalisa variasi genetik antara individu dan untuk mengenalpasti filogeni molekul bagi setiap sampel penyu agar. Kajian ini melibatkan prosedur seperti pengekstrakan DNA genomik, tindakbalas berantai polimerasi, penjujukan DNA dan analisa hubungan filogeni. Sebanyak 510bp dari gen COI telah diperolehi dan digunakan untuk analisa variasi genetik diantara penyu agar. Hanya 0% hingga 0.2% variasi direkodkan. Ini menunjukkan kesemua sampel tersebut tidak mempunyai variasi dan berasal dari leluhur yang sama. Pokok filogeni menunjukkan karakter monofiletik dengan kesemua sampel berkumpul bersama membentuk satu clade.

Kata Kunci: Chelonia mydas, COI, liku-D, Variasi genetik, Filogeni molekul

1.0 INTRODUCTION

Green Turtle (*Chelonia mydas*) is a long lived marine reptile with complex life histories that is extensively distributed in tropical and subtropical regions across the globe. *C.mydas* was named by the green color of the fat underneath its shell or the emerald tint of its muscle. *C.mydas* characterized by a single pair of prefrontal scales which can be utilized to distinguish them with other marine turtles that have two pairs of prefrontal scales. Its carapace can be pale to very dark green or brown, and green tones with radiating stripes while the hatchling of green turtle is dark brown or black in color with white flipper margins. *C.mydas* also known as the herbivorous green turtle (Bowen and Karl, 2007) is appeared to be the only marine reptiles that frequently transfer vital nutrients to the upland coastal habitat by depositing eggs on the beach. As concerned, beach ecosystem has low vegetation and poor nutrient content which then the un-hatched eggs and trapped hatchlings will contribute vital nutrients to the nutrient-poor coastal area. Moreover, green turtles served as the significant strand or important role in the food web of oceanic life.

The genetic structure of *C.mydas* is defined by the geographic allocation of critical habitats and dispersion and its migratory behaviour, in combination with the philopatric nature of the species. Due to the philopatry and natal homing theory, *C.mydas* population was separated into geographically and genetically distinct breeding units that can be identified by using mitochondrial DNA (mtDNA) sequence (Allard *et al*., 1994). In advance, mtDNA control region analyses have been increasingly applied to marine turtles, whereby the development of genetic tags for *C.mydas* has contributed to the acquisition of valuable data on their molecular evolution, population structure, reproductive behavior and migration ecology, besides providing a foundation for conservation and management strategies (Avise, 2007; Bowen and Karl, 2007).

Besides, rapid decline of population number of *C.mydas* has been reported worldwide resulted by the exposure of the species to numerous anthropogenic impacts such as unsustainable coastal development, incidental capture in fishing gear, boat strikes and overharvesting activities particularly in illegal trade. As a result of the declining population number worldwide, green turtle and other marine turtle species have been listed as critically endangered or threatened species by International Union for Conservation of Nature (IUCN, 2010) and also listed in Appendix 1 of the Convention of International Trade of Endangered Species of Wild Flora and Fauna (CITES).

In view of the fact that green turtle has become the quintessential subject for public conservation awareness, this project carried out mtDNA studies to identify genetic divergence and molecular phylogeny of green turtle from two diverse locations in Sarawak coastal area.

Many studies on C.*mydas* from Sarawak had been carried out in term of population size by Tisen and Bali (2000), tagging experiment in assessing turtle migration by Zulkifli *et al*. (2003) and study on green turtle egg hatch rate by Leh (1989). In addition, Yahya (2009) and Tan (2010) had carried out preliminary molecular work on C.*mydas* using D-Loop and 16S gene sequences. However, there is no study yet conducted on C.*mydas* from Sarawak, using Cytochrome Oxidase I (COI) gene sequence. Therefore, this study has been designed with the following objectives. The objective of this study is to obtain the COI gene sequences from *C.mydas* nesting site in Talang-Talang Island and Satang Island. The pattern of mtDNA based on COI gene information was used to assess genetic variation among *C.mydas* in the study site and to infer molecular phylogeny of C.*mydas* based on COI gene information.

2.0 LITERATURE REVIEW

2.1 Marine Turtles and *Chelonia mydas*

Marine turtles can be classified into two families namely Cheloniidae with six species and the Dermochelyiidae with a single highly derived species, the leatherback turtle (Bowen and Karl, 2007). All of the seven species of marine turtle encompass a diversity of ecological niches that can be expressed from the herbivorous green turtle (*Chelonia mydas*) to the spongivorous hawksbills turtle (*Eretmochelys imbricata*) to the oceanic leatherback turtle (*Dermochelys coriacea*) and olive ridley turtle (*Lepidochelys olivacea*) followed by the loggerhead turtle (*Caretta caretta*), Flatback turtle (*Natator depressus)* and Kemp's ridley turtle (*Lepidochelys kempi*) which are coastal carnivores with more cosmopolitan diet (Bowen and Karl, 2007).

Although there is some diversity in the developmental patterns among marine turtles species (Bolten, 2003), in general, hatchling sea turtles leave the natal beach and swim offshore and remain in oceanic habitats for several years. In some populations and species, marine turtles then recruit to a series of neritic habitats, while for other species their oceanic living prevails (Godley *et al*., 2008). This shift in habitat use during ontogeny was then followed by shifts in the diet, from epipelagic omnivorous or carnivorous feeding strategy of the oceanic juveniles to a strong tendency towards herbivory of the neritic stage of green turtles (Carrión-Cortez *et al*., 2010). There were many research has been developed in order to understand the feeding ecology of green turtles (Moran and Bjorndal, 2007).

Besides, period of sexual maturity for C.*mydas* is estimated to be at 26 (Frazer and Ladner, 1986) to 40 years (Limpus and Chaloupka, 1997) subsequent to which they initiate breeding migrations between foraging grounds and nesting areas that are undertaken every few years (Hirth, 1997). During non-breeding periods, the adults of green turtle reside at coastal neritic feeding areas which are sometimes allocated with juvenile developmental habitats (López-Mendilaharsu *et al*., 2005; Carrión-Cortez *et al*., 2010) and for which they display strong site fidelity (Broderick *et al*., 2007).

Marine turtles throughout Oceania have a long history of exploitation and are iconic species of high cultural, traditional, and often spiritual significance to many indigenous groups particularly in the Pacific region (Woodrum, 2010). Thus, marine turtles have become the global conservation concern, which knowledge of their marine environment, migratory habit and their nesting assemblages is essential for effective conservation management and strategies.

Nesting assemblages make up a small proportion of the overall Oceania green turtle nesting aggregation; which they may possess unique adaptations in genetic diversity and ecological significance to their particular environments, and may therefore be important for recovery of the species (Bjorndal and Bolten, 2008). Furthermore, *C.mydas* and other marine turtle species are highly migratory and those that nest in one region are susceptible to threats or causes of mortality in other geographic areas, often with different levels of legislative protection, during different phases of development and migration (Dutton and Squires, 2008).

Populations of C.*mydas* has declined several orders of magnitude on a global scale during the last few centuries (Knowlton and Jackson, 2008) due to direct off take and hunting of all life stages, boat strikes, fatal interactions with fisheries, degradation and loss of feeding and nesting habitats, and ingestion of synthetic material (Campbell and Lagueux, 2005). Population reduction study conducted by Balasz and Chaloupka (2004) showed that the Hawaiian green turtle population was subjected to extensive human exploitation in the form of turtle and egg harvest at foraging and nesting grounds from the mid-1800's until the early 1960's, and nesting habitat destruction as a result of development. Other than that, by catch in fisheries industry has been implicated as a significant source of mortality and subsequent population declines for numerous sea turtle species (Lewison and Crowder, 2007; Chan and Liew, 1996).

Subsequent to rapid population reduction of green turtle and other marine turtle, these marine reptiles have been classified as Endangered by IUCN Red List (IUCN, 2010) therefore several regional and international efforts have focused on reversing its global decline. These efforts include the protection of nesting beaches, banning turtle hunting, installation of Turtle Excluding Devices (TEDs) on fishing gear and the creation of turtle hatcheries (Hays, 2004; Troëng and Rankin, 2005). The success of these efforts is significantly linked to the protection of turtle feeding grounds, mainly the sea grass meadows.

Therefore, comprehensive knowledge of the spatial ecology of these species is critical to allow the identification of key habitats and the likely sources of anthropogenic threats, consequently informing effective conservation strategies (Cooke, 2008).

2.2 *C.mydas* in Malaysia

Out of seven extent species of marine turtles that have been recognized, there are only four species of marine turtles that can be found in Malaysia (Chan, 2006) which are leatherback turtle (*Dermochelys coriaceae*), green turtle (*Chelonia mydas*), hawksbill turtle (*Eretmochelys imbricate*) and olive ridley turtle *Lepidochelys olivacea*). Marine turtles categorized among the better-known sea creatures in Malaysia, with a conservation history dating back to the 1950's. They have been well studied and a great number of scientific studies have been carried out to contribute to the implications for conservation of marine turtles in Malaysia.

C.mydas is widely distributed across Malaysian waters with the most important nesting populations occurring in Sabah and Sarawak Turtle Islands with other nesting beaches can be found in Terengganu (primarily in Redang and Perhentian Islands, Kemaman and Kerteh), Pahang (Chendor and Cherating), Perak (Pantai Remis) and Sipadan Island in Sabah. Moreover, satellite tracking studies have demonstrated that green turtles that nest in Redang Island, Terengganu and the Sarawak Turtle Islands migrate to near-shore feeding grounds occurring in the territorial waters of countries bordering the South China Sea as well as the Sulu-Sulawesi Sea (Bali *et al*., 2002).

A study of habitat use by *C.mydas* nesting site in Peninsular Malaysia further identified some important habitats used by green turtle population in Peninsular Malaysia and the surrounding South-east Asian region using satellite telemetry at the Ma'Daerah Turtle Sanctuary, Terengganu, Malaysia (Merwe *et al.*, 2009) thus enhancing conservation effort for C.*mydas* in Malaysia.

Moreover, history of intensive egg exploitation has been recognized as one of the major causes of green turtle population decline in Malaysia case studies. In Terengganu and Sarawak where hatchery programs have been conducted since 1950's and 1960's, persistent egg harvest for many decades has led to the failure to protect sufficient numbers of marine turtle eggs required for green turtle population maintenance. Government approval on the commercial sale of turtle eggs in the markets in Terengganu have also encouraged smuggling of turtle eggs from places where its sale and places where exploitation have been banned (Chan, 2006).

Other than overexploitation and smuggling of turtle eggs, fishing gear such as trawl nets, drift nets, fish traps, long lines, purse seines, ray nets, lift nets, and even beach seines have been identified to impact sea turtles population in Malaysia (Chan and Liew, 2001). Loss of nesting beaches prior to tourism development primarily at the beachfront area may threaten other existing nesting beaches. The beachfront development also occur in Malaysia where turtle sanctuaries have been established for example Sabah and Sarawak Turtle Islands; Rantau Abang, Ma'Daerah and major nesting beaches in Perhentian and Redang Islands in Terengganu.

Inadequate of management and enforcement of legislation in Malaysia may impact the conservation effort conducted for C.*mydas* population. However, legislation is well-established in Sarawak under the ordinance of Wildlife Protection Ordinance 1998 no exploitation and trade in all marine turtles, their eggs and any derivative or their parts, are prohibited. Besides, legislation in Sabah prohibits commercial exploitation of marine turtles and their eggs (Tisen and Bali, 2000) thus provide certain protection for this species.

In addition, green turtle is an endangered and protected species in Sarawak with primary nesting sites located at the three turtle islands, Satang Besar, Talang-Talang Besar and Talang-Talang Kecil (Leh *et al.,* 1985; Leh, 1989) where population of green turtles from all nesting beaches has estimated to undergo continuous decline in terms of eggs collected, turtle landings and hatchlings emergence (Sarawak Forestry Cooperation, 1996).

In the early 1950's, nesting over twenty thousands per year has been recorded in Sarawak region. However, the nesting trends of green turtle for over the last 30 years appear to be in equilibrium with two to three thousand nesting occurring per year which indicates a decline of over 90% compared to the population number in the 50's (Tisen and Bali, 2000).

According to Harrison (1962), he observed the monthly egg laying cycles of green turtles in Sarawak and noted that the fundamental factor causing the seasonal trend of landing is due to the water temperature associated with the equatorial climate, currents and rainfall. For Sarawak green turtle population, several studies have been conducted which contribute to enhance conservation management of marine turtle primarily green turtle in Sarawak region. Example of scientific research are on the hatch rate of green turtles eggs by Leh (1994), the tagging experiments by Zulkifli *et al*. (2003) and in recent times studies by Yahya (2009), on molecular phylogeny of green turtle using mitochondrial DNA sequence to determine the nesting pattern of green turtle between green turtle population in Sarawak turtle island.

2.3 Molecular studies on *C.mydas*

Studies using molecular approach are increasingly employed particularly in sea turtle conservation efforts. The perception on sea turtle biology and predominantly of sea turtle migration route, nesting assemblages and population structures has increased through genetic approach. Migration route and nesting assemblages of *C.mydas* involved large temporal and spatial scales, which various aspects of green turtle life cycle are quite complicated to clarify by conventional approaches, and must be solved by using indirect research methods, such as molecular genetics (Avise, 2007; Bowen and Karl, 2007).

In addition, proliferation of marine turtle dies in migratory corridors and feeding habitats as a result of a variety of direct and indirect threats, and it remains unknown which nesting colonies are affected by such mortalities. Migratory studies, utilizing electronic tagging systems, have highlighted the likely impacts of threats in wide-ranging species of reptiles (Godley *et al*., 2008) in which the utilization of molecular techniques is much preferred and also offer tremendous insights (Palumbi and Cipriamo, 1998). Besides, current developments in the application of molecular genetic markers can facilitate the identification of origin of marine turtles in migratory corridors or at their feeding habitats (Bowen, 1995b).

Several analyses demonstrate the value of examining mitochondrial DNA (mtDNA) in reviewing genetic structure for conservation management purposes. For instance, the study of female philopatry by Bowen *et al*. (1992) was carried out to explain significant mtDNA haplotype frequency differences among green sea turtles from different nesting beaches.

In addition, subsequent analysis of biparentally inherited nuclear markers revealed male-mediated gene flow and a level of population connectedness that was highly relevant to conservation of the species (Fitzsimmons *et al*., 1997; Roberts *et al*., 2004). In recent times, molecular studies have looked at sampling gaps in the western coast of Africa (Formia *et al*., 2006), and these have facilitated larger-scaled mixed stock analyses (MSAs) to better resolve the movements of green turtles in the Atlantic Ocean (Bolker *et al*., 2007; Naro-Maciel *et al*., 2007). Another molecular studies show that the application of genetics data to conservation management accomplishment is the most powerful in combination with behavioral and demographic information of marine turtles (DeYoung and Honeycutt, 2005). Analyses of individual mitochondrial (mtDNA) or nuclear loci, mitochondrial genome, concatenated data sets, and super matrices have recovered well-resolved tree that concur in many aspects of their topologies and much of the deep phylogeny of extant turtles is now well understood (Thomson *et al*., 2008).

Mitochondrial DNA control region analyses have been increasingly applied to marine turtles, whereby the development of genetic tags for *C.mydas* has contributed to the acquisition of valuable data on their molecular evolution, population structure, reproductive behavior and migration ecology, besides providing a foundation for conservation and management strategies (Avise, 2007; Bowen and Karl, 2007). Moreover, the natal homing behavior of females creates distinct mitochondrial DNA markers at different nesting beaches due to the reproductive isolation of populations (Meylan *et al.,* 1990) and green turtle tend to be morphologically conservative and as a result green turtle has emerged as model organisms for mitochondrial DNA studies (Avise, 2007).

Mitochondrial DNA (mtDNA) was recognized as the molecular marker of choice to detect matrilineal population structure at various geographic scales (Encalada *et al*., 1996) and provide important information on dispersion patterns, geographical variations and phylogeny (Avise, 1986). Sequences of maternally inherited mitochondrial (mtDNA) and microsatellites of biparentally inherited nuclear DNA are the markers commonly used in recent wildlife population genetics (Frankham *et al*., 2002). Hypothetically, due to their faster rate of evolution, the resolution of microsatellites is greater than that of mtDNA sequences. On the other hand, in some cases, population differentiation inferred from mtDNA sequences is stronger than that from microsatellites (Chen *et al*., 2008; Portnoy *et al*., 2010). Thus, this study was conducted by using two different regions of mtDNA genes which are D-Loop region and Cytochrome Oxidase I (COI) gene which will be amplified in order to identify the population structure of green turtle in Sarawak water.

Displacement loop or also known as the D-Loop region, the control region of mtDNA has been described as 0.8kb long and emerge to have control over mtDNA replication and RNA transcription process (Kaska, 2000) which is also recognized as one of the fastest evolving regions and the sequence has turn out to be the best alternative used for origin study and population definition (Bowen, 1995a). Cytochrome *c* oxidase 1 (CO1) gene has been used successfully for species-level identification in several animal groups primarily for vertebrates in which approximately 600 base pair fragment of the mitochondrial DNA sequence will be amplified for genetic studies.

3.0 MATERIALS and METHODS

3.1 Samples Preparation (Adapted from Fitzsimmons *et al*., 1999)

The available green turtle samples collected at the end of 2007 with permit number NPW.907.4.2(II)-92, were utilized for extraction protocol which was in the form of muscle tissues samples and turtle eggs samples. The entire embryo or the soft tissues from advanced embryos have been well-preserved in the DMSO solution with ratio of tissue to buffer between 1:5 and 1:10 (Dutton, 1996). Approximately 2mm tissue samples per C.*mydas* hatchlings was taken from the flipper region by scrapping with a clipper and removed with a scalpel blade from the trailing edge of the carapace. For egg samples, eggshell and egg yolk were separated and collected in a sealed container. The eggshell, egg yolk and the tissue samples were stored in the -80^{0}c ultra freezer.

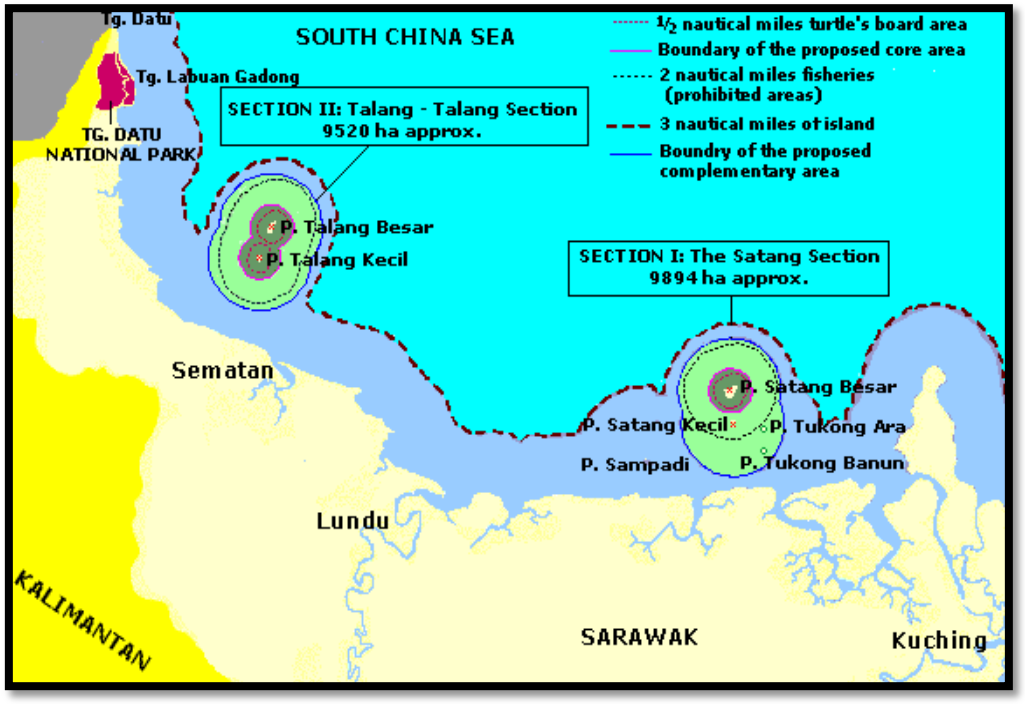

Figure 3.1: Map showing the location of Talang-talang Island and Satang Island, the study site involved in this project.

3.2 Total Genomic DNA Extraction (Modified from Doyle and Doyle, 1987)

For Total Genomic DNA extraction procedure, the preserved tissue samples were minced with autoclaved surgical blade and approximately 500µl of 2x CTAB buffer was added to the tissue samples during the mincing process. Appropriate amount of 0.5g minced samples was placed into 1.5ml Eppendorf tubes which then 5µl of Proteinase K stock solution and 500µl of 2xCTAB buffer were added into the Eppendorf tubes which afterward the tubes was placed in a water bath for incubation at 60°C for about two to three hours. 600µl of Chloroform: Isoamyl alcohol (24:1) was added into the tube and later on, the mixture was centrifuged at 13 000 rpm using "himac CF 15RX" High Speed Micro Centrifuge for 15 minutes at 4°C. The upper aqueous phase or the supernatant was subsequently transferred into a new Eppendorf tube and was mixed with absolute ethanol as the same volume as the supernatant. Several supernatant samples were subjected for incubation overnight in the -20^0C freezer and some samples were placed on the bench for about 15 to 30 minutes. Subsequent to the incubation period, the mixture was centrifuged at 13 000 rpm for 15 minutes at 4°C and the excess ethanol in the tube was removed carefully to avoid the loss of pellet at the bottom of the tubes. The pellet then was mixed with 500µl of 70% ethanol and centrifugation at the same speed, time and temperature was taken place again. Then, excess ethanol was completely discarded from the tubes and the pellet was dried at room temperature for approximately 2 hours. Finally, the pellet was re-suspended in about 50µl deionized water and was stored in -20^0C freezer for long term preservation. The figure below shows the flow chart of total genomic DNA Extraction procedure:

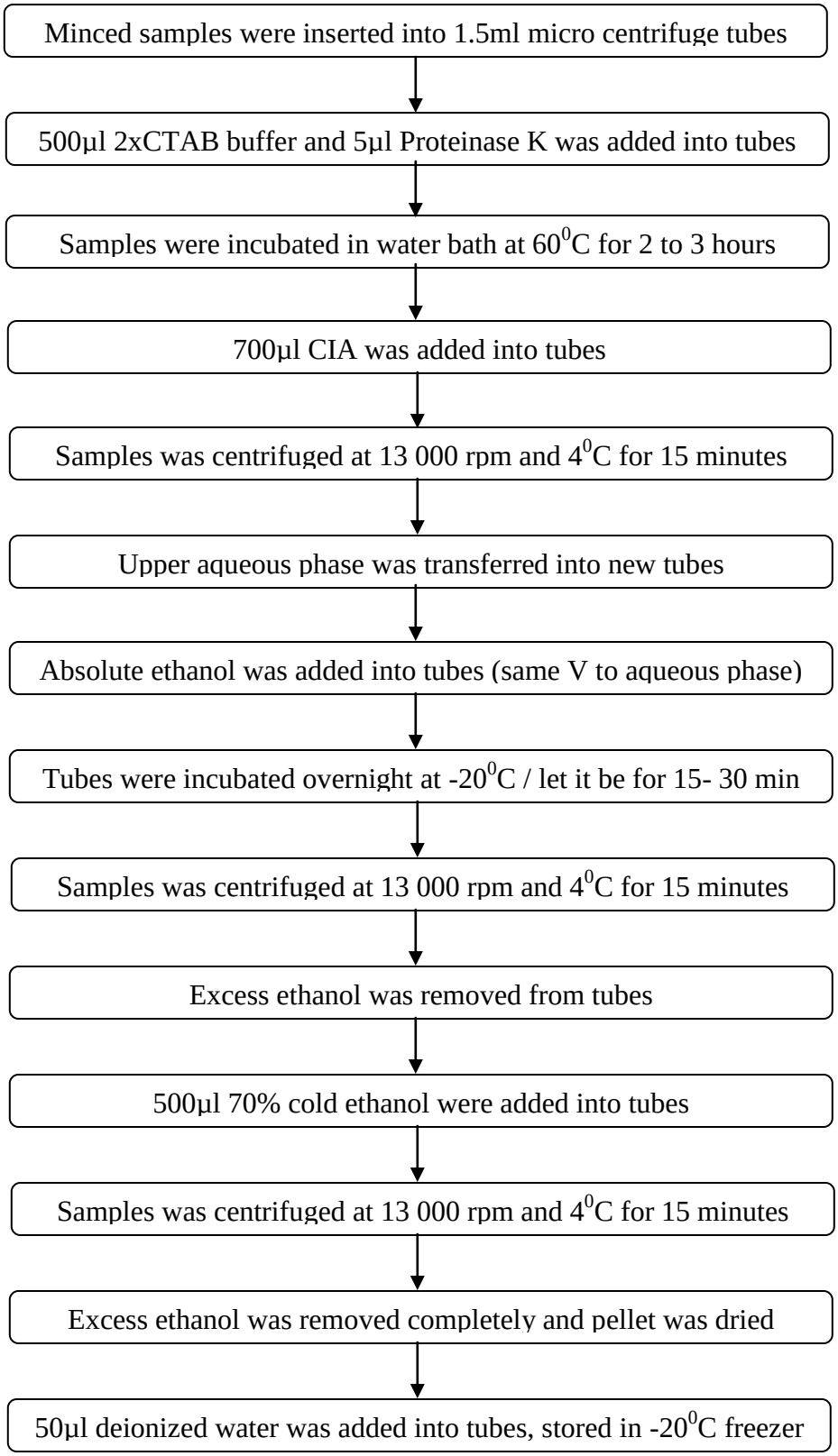

Figure 3.2: Total Genomic DNA Extraction procedure