Faculty of Resource Science and Technology

Establishment of DNA Isolation Protocol from Sarawak Sea Turtle Eggs

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
bp	base pair
cm	centimeter
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	EthyleneDiamine Tetra-Acetic Acid
g	gram
IUCN	International Union for Conservation of Nature
kg	kilogram
mg	miligram
min	minute
mM	milimole
ml	mililiter
$MgCl_2$	Magnesium Chloride
ML	Maximum Likelihood
MP	Maximum Parsimony
NCBI	National Center for Biotechnology Information
NJ	Neighbour- Joining
nm	nanometer
ng	nanogram
PCR	Polymerase Chain Reaction
rpm	revolution per minute
T_a	Annealing Temperature
TAE	Tris acetate Ethylenediaminetetraacetic Acid
TE	Tris- Ethylenediamine Tetra-Acetic Acid
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SFC	Sarawak Forestry Corporation
Taq	*Thermus Aquaticus* DNA polymerase
UV	Ultraviolet
V	Volt

w/v	weight/volume
°C	degree Celsius
%	percentage
μl	microliter
ug	microgram
&	and

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ABSTRACT

Chelonia mydas has been listed in IUCN List as endangered species due to continuous human exploitation. Although there are laws and legislations in protecting this endangered species, turtle eggs are still able to be found sold illegally in the black market in Malaysia. Eggs poachers claimed that the turtle eggs are obtained from foreign countries in order to escape from prosecution. Hence, in this study, three DNA extraction methods have been evaluated in term of DNA yield and amplification quality. Out of three extraction methods, CTAB extraction method produced highest DNA yield from the yolks samples but it is relatively not pure. Besides that, Guanidinium Isothiocyanate Extraction method gives pure DNA but low in concentration. Phylogenetic trees based on D-loop gene sequenced from turtles' eggs showed that it has very close relationship with sample from Satang Island.

Key words: *Chelonia mydas* eggs, Phenol/Chloroform, CTAB, Guanidinium Isothiocyanate, mtDNA, D- loop region

ABSTRAK

Chelonia mydas telah disenaraikan dalam senarai IUCN sebagai spesis terancam. Hal ini adalah disebabkan oleh gejala-gejala manusia yang sering membunuh mereka demi mendapatkan telur and dagingnya. Walaupun terdapat undang-undang yang melindungi mereka, namum, telur penyu masih didapati dijual secara haram di pasar gelap. Kebanyakan, peniaga-peniaga haram itu menafikan bahawa telur penyu itu adalah berasal dari negara kita. Oleh hal yang demikian, dalam kajian ini, sebanyak tiga protocol pengasingan DNA telah diuji dari segi keberkesanan dalam penghasilan DNA yang banyak serta berkualiti. Dari kajian ini, didapati bahawa kaedah CTAB menghasilkan DNA yang banyak tetapi tidak tulen. Walaupun kaedah guanidinium isothiocyanate menghasilkan bilangan DNA yand sedikit, DNA yang dihasilkan adalah lebih tulen berbanding daripada kaedah yang lain. "phylogenetic tree" yang berdasarkan D-loop menunjukkan sampel telur penyu kajian ini adalah berasal dari Pulau Satang, Sarawak.

Kata kunci: Telur *Chelonia mydas*, Phenol/Chloroform, CTAB, Guanidinium Isothiocyanate, mtDNA, D-loop

CHAPTER I

1.0 Introduction

Chelonia mydas or green turtle is one of the most predominant marine sea turtles species that could be found nesting along the coastal beach of Peninsular Malaysia and Borneo (Sabah and Sarawak). However, recent years, rapid developments which turn their nesting sites into tourism spots and continuously exploitation of turtle eggs had lead to great declined of *C. mydas* population. It had been reported that *C. mydas* nesting populations in Peninsular Malaysia were declined by more than 80 % since early of 1950’s (Hendrickson and Alfred, 1961; Siow and Moll, 1981; Limpus, 1993; Ibrahim, 1994; Ibrahim *et al*., 2003). Nearly 100 % of all turtles eggs laid in Peninsular Malaysia were collected for human consumption at a rate of up to 2 million eggs per eggs have also been reported in the late 50’s (Hendrickson and Alfred, 1961; Siow and Moll, 1981). Recently, WWF-Malaysia has reported that about 422,000 eggs were traded in Terengganu in 2007 where it had already exceeded about double the number of *C.mydas* nestings in the state (Turtles face eggs-tinction, 2010).

Strict laws and regulations in protecting marine sea turtles have been enforced in several states in protecting marine sea turtles, but, turtle eggs could still be found sold illegally in the black markets. Permit holders may under report the number of egg collections, or egg poachers may even sneak into the nesting areas to harvest the eggs illegally in order to gain extra income (RM 1.50 – RM 4.00) since turtle eggs are in high demand and high economical value (Noorainie *et al.*, 2009). Besides that, many poachers also claimed that their turtle eggs are obtained from foreign countries such as Thailand, Indonesia, Philippine and China in order to escape from prosecution.

As a result, it is vital to establish a good identification method of the origin of marine sea turtle eggs. Identification of the origin of sea turtle eggs using morphometric technique is often inconclusive due to overlapping egg diameters and nesting ranges between species (Miller, 1997). Eggs cannot be identified using isoelectric focusing (IEF) and fatty acid profiles can only differentiate some species (Seaborn and Moore, 1993). Hence, some other techniques such as DNA extraction methods from turtle eggs are needed to explore and establish to produce high yield and good quality of DNA for further molecular study such as PCR amplification and DNA sequencing. The control region of mitochondrion DNA (mtDNA) which is known as the displacement loop (D-loop), is one of the fastest evolving region and theirs sequence has become the method of choice for origin study and population definition (Bowen, 1997).

In this study, the aims are to explore 3 different DNA extraction methods on different eggs components that could produce high yield and quality of total genomics DNA for further PCR amplification and sequencing. The D-loop region had been amplified and then analyzed to found out the population structure of *C.mydas* around South China Sea geographical region.

CHAPTER II

2.0 Literature Review

2.1 Marine Sea Turtles

Sea turtles are magnificent reptiles perfectly adapted for ocean life. They evolved from land turtles (tortoise) and still share some important features with their distant cousins. Marine sea turtles are the most distinctive creatures among the living reptiles with the presence of hard shells which consists of carapaces (upper shell) and plastron (lower shell) (Lim and Das, 1999). They have waterproof scutes which are made of keratin. Normally, they can be found in tropical and subtropical region throughout the world. Besides that, marine sea turtles have a three-chambered heart and breathe air throughout their live by lungs even though when they are in the sea. Besides that, the most distinct characteristic of sea turtle to be grouped under reptilia classes are their amniotic eggs. The amniote egg contains extra-embryonic membranes that are not part of the embryo and are disposed of after the embryo has developed and hatched.

Marine sea turtles can be further subdivided into several families such as Dermochelyidae, Cheloniidae, Protostegidae, Toxochelyidae and Plesiochelyidae. The latter three families had been reported as extinct taxa long ago (Carl and Roger, 1989). Seven marine turtle's species have been recognized inhabits unevenly in the world's oceans except Arctic. (Pritchard, 1997) Out of this number, only four species can be found nesting on Malaysian shores, they are Green Turtles (*C. mydas*), Hawksbill Turtles (*Eretmochelys* *imbricata*) Leatherback Turtles (*Dermochelys coriacea*) and Olive-Ridley turtles (*Lepidochelys olivacea*) (Chan, 2006).

At present, the family Dermochelyidae is represented by a single present genus, *Dermochelys* (Blainville, 1816) and having the only species, *D. coriacea*. According to Brongersma (1968), this species is the largest living turtle in the world, reaching about 2m long and weighing from 500kg (Pritchard and Tebbrau, 1984) to 916kg (Lim and Das, 1999). The local name for this species is named as Leatherback Turtle. Apart from their colossal size, others distinct diagnostic features that help to identify Leatherback turtle are lacking of horny scutes on adults shells, which is covered with leathery skin (Ernst and Barbour, 1989). Theirs carapace and plastron have seven and five longitudinal ridges (Ernst and Barbour, 1989). Normally, they feed on jellyfish, plankton, crustaceans, mollusks, sponges and others. The nesting sites of these creatures could be found all around the world but only found nesting at coastline of Rantau Abang, Terengganu, between May and September in Peninsular Malaysia (Chan, 2006).

Another family of marine sea turtles, Cheloniidae consists of four genera and six species of sea turtle. They are *Caretta caretta* (Loggerhead Turtle), *C. mydas* (Green Turtle), *E. imbricata* (Hawksbill Turtle) and *L. olivacea* (Olive Ridley Turtle) (Lim and Das, 1999). Out of these, only three species, the Green Turtle, Hawksbill Turtle and Olive Ridley Turtle are known to nest along the shores of Peninsular Malaysia and the first two species nest along the coastline of Borneo (Sabah and Sarawak) (Lim and Das, 1999). Lastly, the fourth species, Loggerhead Turtle has not been known to nest in this region, but is an occasional visitor to West Malaysian waters, as indicated by individuals that are sometimes caught in trawling nest (Lim and Das, 1999).

Figure 2.1: Nesting Sites of Marine Sea Turtles in Malaysia. (Map adapted from Chan, 2006)

2.1.1 Green Sea Turtle (*C. mydas*)

C. mydas are commonly known as green turtles or "Penyu agar" due to its green appearance flesh and have been classified as the family of Cheloniidae. According to Linnaeus (1758), the classification of green turtle is as following:

Kingdom	: Animalia
Phylum	: Chordata
Class	: Reptilia
Order	: Testudines
Family	: Cheloniidae
Genus	: *Chelonia*
Species	: *mydas*
Common Name	: Green turtle
Local Name	: Penyu agar; Penyu hijau; Penyu pulau; Penyu emegit

Green turtles (*C. mydas*) are marine turtles with a worldwide tropical and subtropical distribution and have been listed as endangered species in the IUCN Red List of Threatened Animals (Seminoff and Shanker, 2008). They can be easily distinguished from other types of sea turtles because they have a single pair of prefrontal scales (Figure 2.2). Its head is small and blunt with a serrated jaw. Green turtles hard shells are made of two parts which is carapace (upper part) and plastron (lower part) joined up by a bridge (Ernst and Barbour, 1989). Their carapace is bony without ridges and has four large, non-overlapping lateral scutes while their plastron has four inframarginal scutes (Figure 2.2). Furthermore, the carapace colour varies from pale to dark green while the plastron colour varies from cream white to yellowish (Lim and Das, 1999).

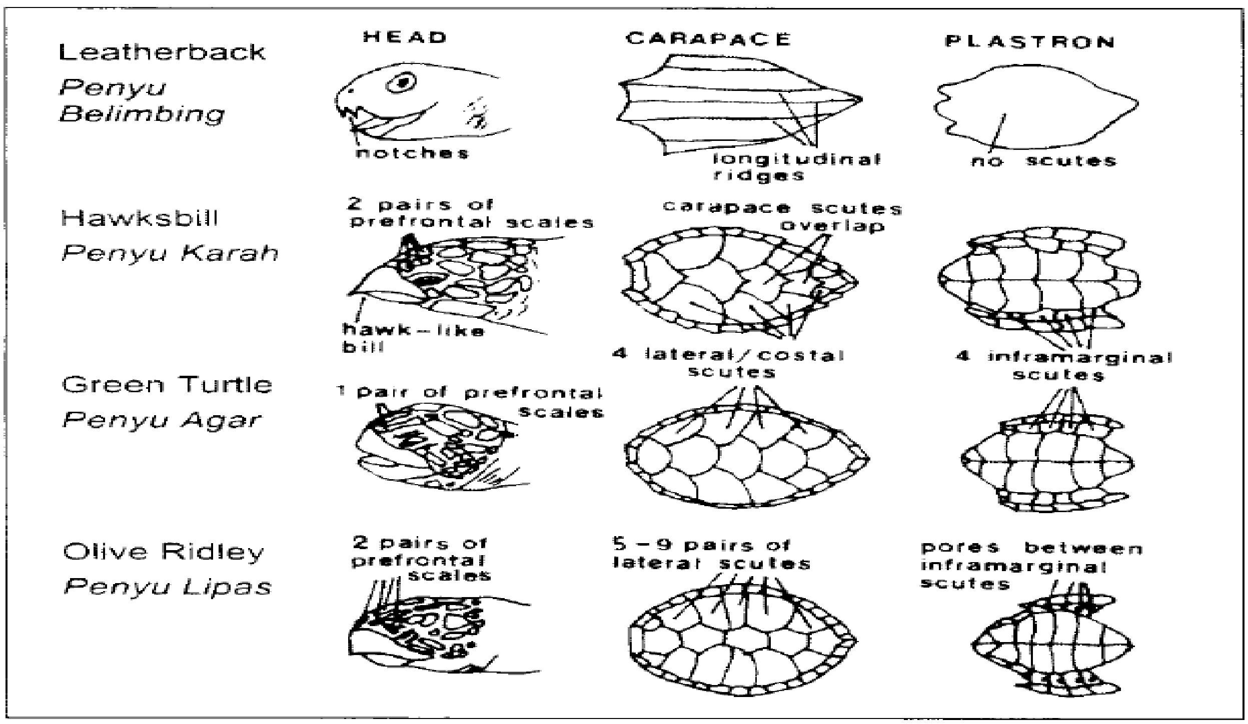

Figure 2.2: Simple identification guidelines of four different species of marine turtle found in Malaysia (Adapted from Beath, 1981; Chan, 2004).

According to Lim and Das (1999), adult green turtle can attain a carapace length of over 140cm and weigh up to 150kg. *C. mydas* is omnivorous; they usually consumed some types of algae, mangrove roots and leaves, sponges, mollusks and jellyfish. Usually, female turtles will return back to their natal beach for laying their eggs nocturnally in order to stay away from predator. Normally, the female green turtles will nest at intervals of 2 – 3 years and lay 4 – 7 nests each season at 10 – 15 days intervals (Ernst and Barbour, 1989). About 100 -150 eggs will be laid in each nest (Ernst and Barbour, 1989). Roughly 50 – 80 days incubation periods (Miller, 1996), the eggs will hatch and new hatchlings will emerge out from the sand in the middle of night where the surface of sand is cool. Emergence of green turtle's hatchlings at night helps to prevent the vulnerable offspring from overheating and visually detected by their predators (Bustard, 1967; Mrosovsky, 1968; Witherington *et al*., 1990; Gyuris, 1993). However, the time spent waiting for the optimal conditions for emergence to occur is certainly not trivial, both in overall energy expenditure (Hays *et al*., 1992) and chances of predation (Godley and Kelly, 1996) or infestation (McGowan *et al*., 2001) can increase dramatically the longer the hatchlings remains in the nest.

Lastly, the sexual determinant of *C. mydas* is temperature-dependant (Janzen & Paukstis, 1991). According to Mrosovsky & Pieau (1991), cool incubation temperatures will produce male hatchlings while warmer incubation temperatures will produce female hatchlings. The sensitive period for sex determination appears to occur around middle of the incubation. The threshold temperature which have been variously referred to as the 'threshold' by Bull (1980), 'pivotal' by Mrosovsky and Yntema (1980), 'critical' by Pieau (1976), and

‘SDT_{50}’ by Limpus *et al*. (1983) for the transition from the production of 1:1 sex ratio is around 28 ^{0}C – 30 ^{0}C for all turtle species (Ackerman, 1997).

2.2 Green Turtle (*C. mydas*) Eggs

Green turtle eggs are type of amniotic eggs that contain three different components which is albumen, yolk and shell. These types of amniotic eggs have a tough outer shells and membranes to protect the embryo from drying out. Besides turtles, others reptiles such as crocodiles, snakes and alligators or even birds possessed amniotic eggs. According to Fischer *et al.*, (1991), oviparous reptiles characteristically allocate the majority of their total reproductive investment to their eggs, thus, similar happened to turtles. Currently, there is no preliminary study on the composition and component of *C. mydas* eggs, but, there are several researches on component of other type of turtle eggs. For examples, a study have been conducted by Wang *et al*., (1997) on the eggs components and utilization during incubation in the turtle, *Chinemys reevesii* can be used as reference or guide. An amniotic egg consists of four basic structures which are yolk and its associated membranes, germinal disc, albumen and its shell with its associated membranes (Rebecca, 2010). Each component performs specific functions in the development of the embryo.

All marine turtles lay white, spherical cleidoic eggs with flexible calcareous shells (Miller, 1985). The sizes of eggs laid in each clutch varies slightly between clutches laid by one female as well as considerably within and between species (Hirth, 1980; Van Buskirk and Crowder, 1994). *D. coriacea* are among all marine turtles lays the largest eggs (5.3cm, 90g), but *Natator depressus* produces eggs that are nearly the same size (5.1cm, 80g). While *C. mydas* lays medium large eggs (4.5cm, 48g), followed by *C. caretta* eggs (4.0cm, 36g); *E. imbricate* lays small eggs (3.8cm, 28g), *L. olivacea* lays eggs of similar size (3.9cm, 35g; 3.8cm, 30g, respectively) (Van Buskirk and Crowder, 1994). However, these morphometrics techniques are inadequate in identifying the origin of marine sea turtles.

Unlike mammals, the offspring of turtles are not fed by their mother during their development. Their mother will excavate a deep hole, laid them into the holes and buried them with sand in the sandy beach. The incubation temperature is much depends on heat of sun. Hence, for their developments, sufficient enough of nutrition and mineral is necessarily in their embryonic developmental stage. Consequently, the egg yolk provides vital nutrients and sources of energy for the survival and growth of the turtles' hatchling. The yolk content declined as the incubation time and embryo mass increased (Wang *et al*., 1997). This phenomenon reflects a potential relationship between yolk consumption and embryonic development. Normally, the major yolk content is lipid where it provides an embryo with greater amounts of usable metabolic energy and water (Gutzke and Packard, 1987) and most of it has been transferred to their hatchling for it cost in the first days after it leaves the egg (Fischer *et al*. 1991).

Turtle eggshells consist of two types which are flexible-shell and brittle-shell (Mitrus, 2003). Each types of eggshell have three layers which are the outer layer (calcareous layer), the middle layer (crystalline layer) and the inner fibrous layer (Bishop, 1998). Besides that, the fibrous layer of *C. mydas* eggshells consists of both aragonite form and calcite form of $CaCO_3$ (Solomon and Baird, 1976; Baird and Solomon, 1979). At day 3 of ovulation, the green turtle eggs are already contained slightly calcified shell membrane (Miller, 1982). Thickness of shell will then rapidly be increased until 8 – 9 days of post – ovulation and are ready for oviposition (Miller, 1982). However, during incubation periods, the shell mass in the eggs will declined as the incubations days increased. This is because during the embryonic developments, the insufficient supply of calcium and magnesium in the eggs contents have been replaced by the calcium and magnesium reservoirs in the eggshell (Wang *et al.*, 1997).

2.3 Current Status of Sea Turtles

Contemporary, sea turtle populations had been declined tremendously worldwide and driven toward extinction. Over three generations of sea turtles, the Marine Turtle Specialist Group had revealed that a total of 94% of declination in populations in the 2004 Global Status Assessment. In Malaysia, the marine sea turtles nesting trends also showed a tremendous decline except for the Sabah populations. The most dramatic declines are exhibited by the leatherbacks, hawksbills and olive ridleys of Terengganu where current nesting numbers indicate that these species are virtually extinct (Chan, 2004). Furthermore, the leatherback population has declined from 10,000 annual nesting in the early 50's to less than half in recent years (Chan and Liew, 1996; 2001).

In Sarawak, the green turtles nesting trend at Pulau Talang and Pulau Satang Island over the last 30 years appear to be in equilibrium, with two to three thousand nesting occurring per year (Chan, 2004). However, as compared to the early 1950's where over 20,000 nesting per year have been recorded, this indicated that about 90 % of declination of sea turtle populations in Sarawak currently (Tisen and Bali, 2000). Only the green turtle populations of the Sabah Turtle Islands have staged a recovery, with current annual densities of over 8,000 nesting representing a threefold increase over levels recorded in the early 1980's (Chan, 2004).

There are several factors that caused sea turtles populations in Malaysia declined. One of the major causes is the intensive egg exploitation where a number of marine turtle species have been decimated. Although there are some hatchery programs in Terengganu and Sarawak, continued egg harvest for many decades has led to the failure to protect sufficient number of eggs required for turtle population maintenance. Government sanctioning of egg collection through issuance of licenses and legal sale of turtle eggs in the markets of Terengganu to this day has not only continued to jeopardize conservation programs, but has encouraged smuggling of eggs from places where its sales and exploitation have been banned.

Furthermore, local communities of neighbouring countries such as the Philippines, Vietnam, Thailand in hunting and slaughtering marine sea turtles for theirs meat and shells had caused the local populations to decline since marine sea turtles are highly migratory. Satellite tracking studies had 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 and Sulu-Sulawesi Sea (Liew et al., 1995; Bali et al., 2002). Besides that, incidental captures in fishing gears (trawl nets and drift nets) had been reported that about 50 – 100 turtles drown per year (Chan and Liew, 2002).

In addition, over-development of nesting sites for tourism had destructed the turtle feeding ground (seagrass bed and coral reefs) and habitats which rendered them unsuitable for nesting. Human activities such as coastal development and some artificial lighting from tourist had disturbed the sea turtles from landing successfully to nest their eggs. Pollution results from discarding solid toxic wastes into sea can further degrade their feeding ground and have impact on them. Sometimes, sea turtles may accidentally ingested plastic debris such as balloons, bottles, vinyl films, and styrofoam floating on the surface of seas had lead to death of sea turtles. Recently, a group of Australia researchers had successfully proved that the level of chemical contamination in green turtles' eggs was considerably high until it might create serious health risks to humans who consume the *C. mydas* eggs (Jason *et al.*, 2009).