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GENETIC STRUCTURE AND DIVERSITY OF GREEN TURTLES (*Chelonia mydas*) FROM TWO ROOKERIES IN THE SOUTH CHINA SEA

JUANITA JOSEPH*[1] AND HIDEAKI NISHIZAWA[2]

[1]*Institute of Oceanography and Environment, Universiti Malaysia Terengganu, Terengganu, Malaysia.* [2]*Graduate School of Informatics, Kyoto University, Yoshida Honmachi, Sakyo-ku, Kyoto 606-8501, Japan.*

**Corresponding author: juanita@umt.edu.my*

Abstract: To understand the spatial and temporal genetic structure of green turtle (*Chelonia mydas*) in South China Sea, we analyzed 178 samples collected from two Malaysian rookeries (Redang Island and Sarawak) using the 800 base pair mitochondrial DNA (mtDNA) control region sequences. Nucleotide diversity in Redang Island and Sarawak was 0.027 ± 0.014 and 0.023 ± 0.011, respectively. The results confirmed the spatial genetic differences between the two rookeries, and supported the natal philopatry of turtles in South China Sea. However, comparison with 20-year-old data from previous study showed no significant temporal differences, indicating that the genetic structures of the populations are temporally stable. The two major rookeries for the green turtles in this study are different in their genetic make-up, thus should be treated as different management units to ensure their future survival.

Keywords: *Chelonia mydas,* Malaysia, sea turtle, natal homing, genetic diversity.

Introduction

The major problems in management of sea turtles include recognising genetically and demographically discrete stocks, as well as to protect and manage these stocks over the vast geographic range occupied during their life. Sea turtles unique life history. They are highly migratory and undertake complex movements and migrations through geographically diverse habitats (Hirth, 1997; Miller, 1997). Despite being highly migratory and occupy vast geographical area, tagging studies show strong segregation of females from different nesting beaches (Limpus *et al.*, 1992). These discrete reproductive populations appear to be shaped by the natal homing behaviour (Nishizawa *et al.*, 2011; 2016). Because only females ascend nesting beaches, biological information has come mainly from tagging of nesting females. In recent years, molecular genetic markers are used to uncover aspects of the life history and evolution of marine turtles, as well as a priority research in turtle recovery plans (Moritz *et al.*, 2002; Bowen & Karl, 2007).

The tropical waters of Southeast Asian region support many green turtle nesting populations, as well as feeding assemblages, and are of global significance for sea turtle populations (Moritz *et al.,* 2002). The green turtle is listed as Endangered (IUCN 2015), and protected marine animal under CITES, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (Groombridge & Luxmore 1989). The swimming ability of green turtles suggest that their genetic population structures may be homogenous over wide geographic range, but in fact their genetic population structures have been reported to be differentiated among rookeries, because of natal philopatry (reviewed by Bowen & Karl, 2007). An understanding of the population genetic structure of a species is required to establish effective conservation measures and management policies, and for the setting of management units (Moritz *et al.*, 2002). In a previous study, Dethmers *et al.* (2006) investigated the genetic structure of 27 Australasian green turtles populations, and revealed significant genetic differences between these rookeries. However, their study was based on shorter sequences (ca. 380 bp) of mitochondrial DNA (mtDNA), rather than those now generally utilized for sea turtle molecular

studies (e.g. LeRoux *et al.*, 2012; Shamblin *et al.*, 2012; 2014). Longer sequences increase the resolution of the results, and thus have been capable of differentiating one haplotype into several discrete haplotypes that were previously assumed to be composed of only a single one. Additionally, because Dethmers *et al.* (2006) analyzed samples collected in 1993 and 1991, the analysis of newly collected sea turtle samples will contribute to the understanding of the temporal variation on a decadal time scale.

Other than the study of Dethmers *et al.* (2006), Joseph (2006) had also conducted a genetic population study of sea turtles in Malaysia using microsatellite DNA markers. The findings had also shown significant differences between the nesting beaches in Malaysia. Apart from that, using microsatellite DNA markers, paternity study of sea turtles in Malaysia had also been determined (Joseph & Shaw, 2011).

This study aimed to determine the genetic population structures of the Malaysian green turtle rookeries in the South China Sea and to analyze (1) spatial genetic differences between the two Malaysian rookeries, and (2) temporal genetic differences on a decadal time scale.

Materials and Methods

Sample Collection

Samples were collected in 2014 (March – September) from the hatchlings of identified (tagged) nesting green turtles in Redang Island (05°49'N, 103°00E) and Sarawak (01°54.7303' N, 109°46.6260' E) (Figure 1). Because of matrilineal inheritance, hatchlings are assumed to contain the same mtDNA haplotypes as their mothers. Only one hatchling was collected from each nest. Blood samples were collected and preserved in a lysis buffer following Joseph *et al.* (2016). A total of 56 and 122 samples from different nesting green turtles in Redang Island and Sarawak, respectively, were analyzed. Sampling of sea turtles was conducted under the permits NCCD.907.4.4 [Jld. 9] – 67 and Export Permit No. 15017 to transport the blood samples from Sarawak to Universiti Malaysia Terengganu.

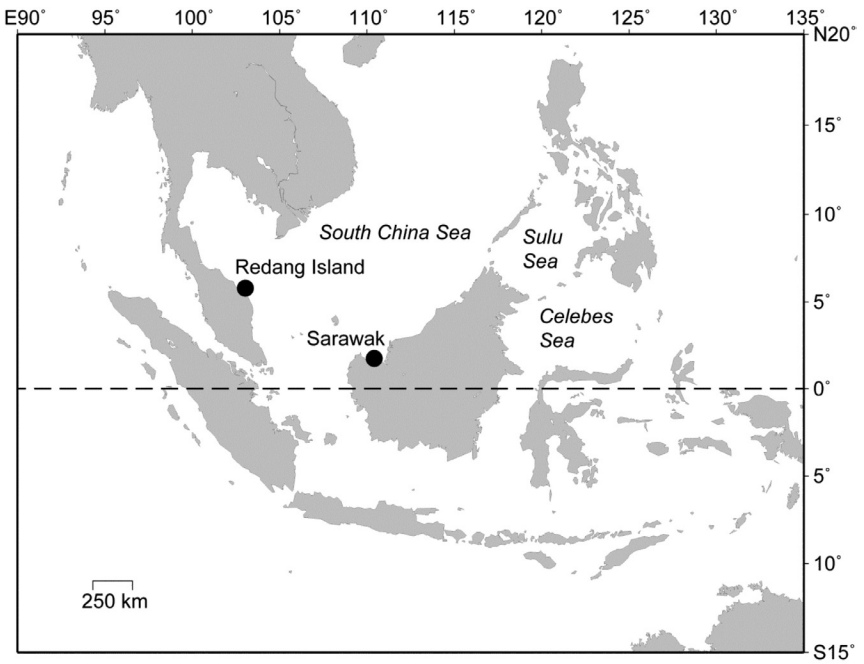

Figure 1: Rookery collection sites for the green turtle samples (Redang Island and Sarawak) in South China Sea. Map created using Maptool program of SEATURTLE.ORG

Laboratory Analysis

Genomic DNA was extracted using a quick CTAB protocol (Bruford *et al.,* 1992) and was amplified using the 800bp primers LCM15382 and H950g (Abreu-Grobois *et al.* 2006). The polymerase Chain Reaction (PCR) amplification and sequencing protocol were based on Joseph *et al.* (2016).

Data Analysis

Sequences were checked using CLUSTALW (Tamura *et al.*, 2013). Haplotypes were identified by performing a search against a collated database of known green turtle haplotypes. The Southwest Fisheries Science Center, NOAA Fisheries Service (https://swfsc.noaa.gov), and the GenBank database (National Center for Biotechnology Information, USA: NCBI website http://www.ncbi.nlm.nih.gov) was referred to the Pacific and Indian Ocean green turtle mtDNA sequences.

An unrooted network of detected haplotypes was created using TCS v1.21 (Clement *et al.*, 2000). Nucleotide diversity (π) and haplotype diversity (h) were estimated using ARLEQUIN v3.5 (Excoffier & Lischer, 2010). To identify the genetic differentiation between Redang Island and Sarawak rookeries, haplotype frequency was compared using the exact test implemented in ARLEQUIN v3.5. In addition, the significance of Φ_{ST} and conventional F_{ST} values was tested via a permutation test (10,000 permutations) in ARLEQUIN v3.5. The nesting green turtles of Redang Island (n = 12) and Sarawak (n = 22), sampled in 1993 and 1991, respectively, had been previously analyzed by Dethmers *et al.* (2006). Therefore, differences in haplotype frequency, Φ_{ST} significances, and conventional F_{ST} values between this study and that of Dethmers *et al.* (2006) were also tested to analyze the temporal consistency of the populations' genetic structures. Because Dethmers *et al.* (2006) identified haplotypes using shorter sequences, sequences acquired in this study were trimmed for these comparisons.

Results and Discussion

A total of eight haplotypes were identified from the Redang Island and Sarawak rookeries. Six haplotypes were found in each rookery, of which four haplotypes were shared between both rookeries (Table 1). Five haplotypes including the four shared haplotypes, CmP49.1, CmP 57.1, CmP 82.1, CmP87.1, and CmP 91.1, contained sequences identical to those haplotypes based on shorter gene regions, from rookeries in Southeast Asia and Australia (Dethmers *et al.*, 2006). Haplotype CmP49.1 was observed at a relatively low frequency (4 of 70 samples) in the northwestern Pacific rookeries of Central Ryukyus, Japan (Hamabata *et al.*, 2014). In addition, the haplotypes CmP49.1 (1 of 48 samples) and CmP91.1 (1 of 538 samples) which had been previously observed in the western Pacific rookeries of Guam/Commonwealth of the Northern Mariana Islands and Ulithi Atoll of Federated States of Micronesia, respectively (Dutton *et al.*, 2014) were also observed albeit at low frequencies. One haplotype (CmP133.1) has not been reported in previous studies, and

Table 1: Haplotype frequency of green turtles in Malaysian rookeries

Haplotype	Redang Island	Sarawak	Haplotype of shorter sequence in Dethmers *et al.* (2006)
CmP49.1	28	26	C3
CmP57.1	0	1	D2
CmP82.1	3	5	B5
CmP87.1	6	87	C4
CmP91.1	2	0	C14
CmP103.1	7	0	
CmP104.1	10	2	
CmP133.1	0	1	
Total	56	122	

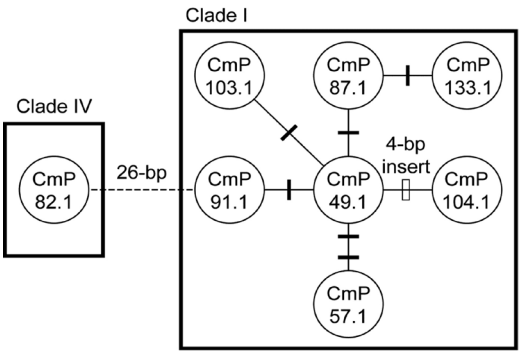

Figure 2: Network of detected haplotypes. A black bar indicates one base substitution. Names of clades correspond to those of Dethmers *et al.* (2006)

was thus registered in Genbank (accession no. KP893544). The unrooted network of haplotypes (Figure 2) indicated that the haplotypes belonged to two clades, corresponding to clades I and IV as described by Dethmers *et al.* (2006). Nucleotide diversity (π) and haplotype diversity (h) were $\pi = 0.027 \pm 0.014$ and $h = 0.699 \pm 0.051$ in Redang Island, respectively, and $\pi = 0.023 \pm 0.011$ and $h = 0.448 \pm 0.046$ in Sarawak, respectively.

Significant differences between the Redang Island and Sarawak rookeries were demonstrated by differences in haplotype frequency ($p < 0.00001$) and F_{ST} value ($F_{ST} = 0.313$, $p < 0.00001$), but not the Φ_{ST} value ($\Phi_{ST} = 0.024$, $p = 0.09$). Dethmers *et al.* (2006) indicated that the mtDNA haplotypes of green turtles in Southeast Asia and western Pacific green turtles were divided into several clades that were supported by the analysis of longer sequences of data by Dutton *et al.* (2014). In agreement with Dethmers *et al.* (2006), haplotypes in two distinct clades (Clades I and IV in Dethmers *et al.*, 2006) were also observed at the South China Sea rookeries in this study. While Clade IV haplotypes were more variable and common in the northwestern Pacific, especially in the central Ryukyus (Hamabata *et al.*, 2014), the coexistence of the two distinct clades is suggested to be the characteristic of green turtle rookeries in the South China Sea. In addition, the significant genetic differences between the two rookeries in this study confirmed the natal philopatry of green turtles in South China Sea. Tagging programs conducted by the Universiti Malaysia Terengganu at Redang Island since 1993 (Chan, 2013) and the Sarawak Forestry Corporation since 1996 (Sarawak Forestry Corporation, unpublished data) had also shown nesting site fidelity of female turtles to these rookeries with yearly return to the same beach for nesting. This study confirmed the genetic differences between Redang Island and Sarawak rookeries hence it is suggested that these rookeries should be treated as different management units. The heterogeneity among nesting rookeries of green turtles in the South China Sea suggests that recently depleted populations cannot be restored through natural colonization, except over a very long-term period (i.e. 100 or 1000's of generations). The long-term sea turtle conservation efforts conducted by the Universiti Malaysia Terengganu, Fisheries Department of Malaysia and the Sarawak Forestry Corporation should be continued to ensure high hatchlings output to replenish future stocks of these two rookeries.

Comparisons with the study by Dethmers *et al.* (2006) detected no significant differences between the Redang Island data from this study and that of the previous study (exact test: $p = 0.20$; $\Phi_{ST} = 0.085$, $p = 0.07$; $F_{ST} = 0.016$, $p = 0.25$). The same was true for the Sarawak rookery (exact test: $p = 0.52$; $\Phi_{ST} = -0.023$, $p = 0.57$; $F_{ST} = 0.007$, $p = 0.26$). Thus, in contrast to spatial genetic differentiation, no temporal genetic differentiation was detected for the Redang Island and Sarawak rookeries. The similarity in haplotype compositions from this study (sample collected in 2014) to those recorded by Dethmers *et al.* (2006) (samples collected in 1993 and 1991) indicated that the populations' genetic structure has been consistent over the past 20 years. Nishizawa *et al.* (2016) investigated the population's genetic structure of the hawksbill turtle samples from Southeast Asia and reported that no temporal genetic changes were found to have occurred on a decadal time scale. Other studies had also reported no genetic differences on a decadal time scale in sea turtle populations (Bjorndal *et al.,* 2005; Shamblin *et al.,* 2011),

despite the restricted resolutions resulting from having a shared dominant haplotype (Bjorndal *et al.,* 2005), or the apparent temporal differences observed in some rookeries (Shamblin *et al.,* 2011). Strong genetic drift is estimated to cause temporal genetic heterogeneity, but the overlapping of turtle generations resulting from long generation times (8–12 years in captive turtles: Bjorndal *et al.* 2013; and an estimated 25–50 years in wild turtles: Chaloupka & Limpus, 2005) and a long turtle reproductive period (estimated to last about 19 years after reaching maturity, according to Chaloupka & Limpus, 2005) probably contribute to the temporal genetic homogeneity observed in the green turtle populations examined in this study. At the same time, newly recruited nesting turtles possibly have similar genetic compositions, confirming the presence of little gene flow between differentiated populations on a decadal time scale, and natal philopatry.

Conclusion

In conclusion, this study has confirmed the presence of spatial genetic differences between the two Malaysian rookeries of Redang Island and Sarawak, a further evidence for the natal philopatry of turtles. However, no significant temporal differences in the Malaysian green turtle populations on a decadal time scale were observed. The two major green turtle nesting rookeries in South China Sea are different in their genetic make-up and must be locally conserved and managed to ensure future survival of the populations. The long-term protection of sea turtles and their eggs at nesting beaches as currently conducted at Redang Island by Universiti Malaysia Terengganu and Fisheries Department of Malaysia, and Sarawak by the Sarawak Forestry Corporation is an effective and essential conservation strategy.

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