No genetic divergence of green turtle Chelonia mydas nesting populations between the Andaman Sea and the Gulf of Thailand

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ABSTRACT

Nucleotide sequences from the control region of the mitochondrial (mt) DNA were analyzed for Thai green turtles (*Chelonia mydas*) to reveal population genetic structure. Four primers were employed *i.e.* Green15552F (GTGTC CACAC AAACT AACTA CCT), Green16300R (GTCTC GGATT TAGGG GTTTG GCG), Green15579F (CTGCC GTGCC CAACA GAACA), and Green16087R (CCAGT TTCAC TGAAT CGGCA). The aligned sequences contained 438 base pairs (bp) with 254 polymorphic sites. There were 8 haplotypes assigned from the 49 green turtles sampled from the Andaman Sea (19 individuals) and the Gulf of Thailand (30 individuals). Low genetic divergence between the nesting green turtle populations of the Gulf of Thailand and the Andaman Sea was detected in both haplotypic (Gst=0.00311) and nucleotide levels (Nst=0.02838) as well as genetic distance (DTN=0.016±0.003). Haplotype frequencies were not significantly different between the two nesting sites. The result was in contrast with a finding using satellite telemetry that discovered separated home ranges. Recent population separation and/or highly conservation of the studied mtDNA region might be an explanation.

INTRODUCTION

Thailand faces two seas *i.e.* the Gulf of Thailand and the Andaman Sea. The two seas are separated by the southern part of Thailand through Malaysian peninsular and further semi-separated by Indonesia (Fig. 1). These geological barriers act effectively to limit geneflow among conspecific populations from the two seas as revealed in several marine organisms *e.g.* banana prawn *Peneaus monodon* (Supungul *et al.*, 2000; Klinbunga *et al.*, 2001), giant clams *Tridacna squamosa, Tridacna maxima* (Kittiwattanawong, 1999; Kittiwattanawong *et al.*, 2001), starfish (Benzie, 1999), rock oysters *Crassostria spp.* (Bussarawit, 2003). Additionally, separation at community level was detected (coral reef fish communities, Satapoomin, 2002). At the larger scale, this geological barrier may serve as a wall to separate marine organisms between Indian and Pacific oceans.

A green turtle *Chelonia mydas* is another organism distributes in both the Gulf of Thailand and the Andaman Sea (Phasuk, 1992). This allows a possibility to test the effectiveness of this geological barrier. In our previous study (Kittiwattanawong *et al.*, In press), satellite transmitted tracking of the nesting green turtle populations from the Andaman Sea and the Gulf of Thailand suggested contemporary allopathic life cycles (Fig. 1). However, such a finding only reflects the present scenario. An improved understanding of life history may be obtained by research on population

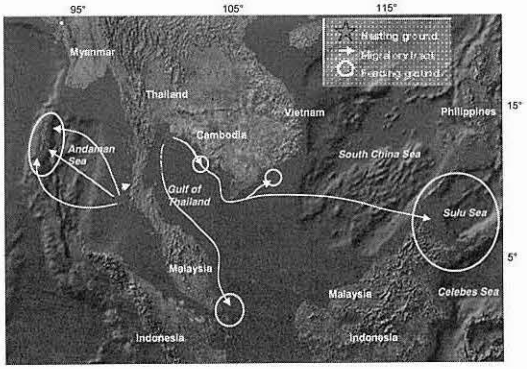

Fig. 1. The two major nesting grounds of green turtles Chelonia mydas in Thailand (Khram Island in the Gulf of Thailand and Huyong Island in the Andaman Sea) with the satellite transmitted results showing the simplified migratory routes and their feeding grounds.

genetic structure. Several kinds of genetic materials vary from proteins to nucleic acids can be employed to reveal population genetic structure (Avise, 1994). Since, genetic materials are inherited from one generation to another, information obtained from genetic materials reflect the summary of natural history from the past till present (Futuyma, 1986; Page and Holmes, 1998).

This study is an analysis of nucleotide sequence from mitochondrial DNA (mtDNA) at control region or D-loop which is recognized as highly polymorphic site (Norman *et al.,* 1994). Within the d-loop, the mutation rate is approximately five to ten times that of the rest of the mitochondrial genome, (Aquedro and Greenberg, 1983). The paper describes the genetic diversities and divergence of the two green turtle nesting sites i.e. Khram Island in the Gulf of Thailand and Huyong Island in the Andaman Sea (Fig. 1).

MATERIALS AND METHODS

The tissues

The samples of turtles tissues were collected with supports from the Royal Thai Navy during 2001-2002. Usually, the staffs patrolled the beaches at night during high tide. After a turtle had laid eggs, the staffs scanned for a microchip tag at both flippers, and a new one would be inserted,subcutaneously to the left flipper when it was not found. Thereafter, a small piece of skin tissue (approximately 0.3x0.3 cm^2) at the inner flipper was cut with a sterile surgery knife and put into a 2-ml microcentrifuge tube filled with sodium chloride saturated DMSO or TNES (a mixture of 150 mM NaCl, 10 mM Tris-HCl pH 7.5-8.0, 25 mM EDTA, and 0.5% SDS) solutions and stored at room temperature. The wounds were treated with medicine such as Gentian Violet, Povidoiodine, or tetracycline ailment before the turtles were released. Twenty-seven samples were collected from Khram Island in the Gulf of Thailand and nineteen samples were from Huyong Island in the Andaman Sea. All samples were analyzed at Graduate School of Agriculture, Kyoto University, Japan under the permission of the CITES.

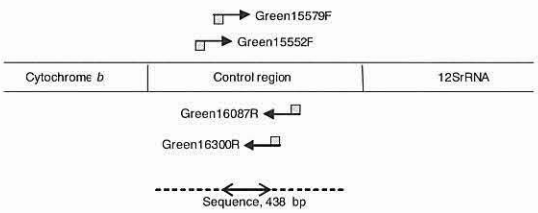

Fig. 2. Location of the primers employed in the study overlaid to a none-scale mtDNA of Chelonia mydas. Arrows indicate the nucleotide synthesis directions. The line with both ends arrows indicates proximal length of nucleotide sequence (438 bp).

DNA analysis protocol

The tissues were digested with Proteinase K. The DNA solutions were obtained by a standard phenol/chloroform extraction (Sambrook *et al.,* 2001) and precipitation. The forward primers *i.e.* Green15552F (FGTGT C CACA CAAAC TAACT ACCT), Green15579F (CTGCC GTGCC CAACA GAACA) and reward primers i.e. Green16300R (GTCTC GGATT TAGGG GTTTG GCG), Green16087R (CCAGT TTCAC TGAAT CGGCA) were used to obtain the specific nucleotide sequences in the control region mtDNA (Fig. 2). Afterward, the selected sequences were amplified with a PCR machine. The PCR products then were run on the Argarose gels to identify the successful of PCR amplification. Finally, the PCR products were sequenced with an automated sequencer to obtain direct reading of the nucleotide sequences.

Data analysis

The nucleotide sequences were aligned and cut using the computer program CLUSTAL W 1.7 multiple sequence alignment (Thompson *et al.,* 1994). Haplotypes were determined by examining the aligned sequences. Haplotypes were assigned when one or more base changes differed from the consensus or conserved sequence. Haplotype (h) and nucleotide (π) diversities were calculated according to the method described by Nei 1987. Divergences between the two populations were calculated as Gst (based on haplotype frequencies, Hudson *et al.,* 1992), Nst (based on nucleotide sequences, Lynch and Crease, 1990), and Tamura-Nei's genetic distance (DTN, Tamura and Nei, 1993). Nst is similar to Fixation indices (Fst) described in Weir and Cockerham (1984), but uses the Jukes and Cantor (1969) correction. Additionally, the differences in haplotype frequency among populations and the nucleotide divergence among haplotypes are also taken into account in the calculation of Nst (Ramey II, 1995). Gst and Nst values range from 0 to 1, which indicate from non existence of population subdivision to well defined sub population. A chi-square test (Hudson *et al.,* 1992) based on pair-wise comparisons of haplotype frequency data was also conducted to detect genetic differentiation between populations. Geneflow (Nm) between the two populations was estimated from Nst and Gst values by the formula Nm=0.5(1/Nst or Gst-1) (Wright, 1951). Nm can be interpreted as the absolute number of individuals exchanged between populations per generation (Avise, 1994). All calculations were performed by the program DnaSP version 3.99.5 (Rozas and Rozas, 1999) and MEGA version 2.1 (Phylogenetic and molecular evolutionary analyses, Kumar *et al.,* 2001). The nucleotide sites with gaps or missing data were completely excluded from the analysis. All sampling errors were reported as standard error (SE) calculated by the mentioned programs with 1,000 bootstrap replicates (Nei and Kumar, 2000). A chi-square test was conducted to test for a significant genetic divergence between the two populations (Nei, 1987; Hudson *et al.,* 1992).

RESULTS

Diversity

The aligned sequences contained 438 base pairs (bp) with 254 polymorphic sites. There were 8 haplotypes assigned from the 49 green turtles sampled from the Andaman Sea and the Gulf of Thailand (Table 1). The two most dominant haplotypes (B1 and A1) were observed in common in the both waters. The number of haplotypes was higher in the samples from the Gulf of Thailand (7 haplotypes *i.e.*

A1, A2, A3, B1, B3, B4, B5, and B6) than the Andaman Sea (3 haplotypes *i.e.* A1, B1, and B3). The haplotype A2, A3, B4, B5, and B6 were detected only from the Gulf of Thailand, while B3 was edemic to the Andaman Sea.

Overall haplotype diversity (Andaman Sea and Gulf of Thailand combined) for the green turtle nesting populations of Thailand was high (h=0.640; Table 2). However, haplotype and nucleotide diversities in all cases might be slightly less than the actual value since the calculations excluded gaps in the aligned sequences. The Gulf of Thailand had a slightly higher degree of haplotype diversity than the Andaman Sea. On the contrary, nucleotide diversity was higher in the population from the Andaman Sea than the Gulf of Thailand.

Table 1. Distribution of the mtDNA control region haplotypes between the nesting populations of the Andaman Sea and the Gulf of Thailand.

Haplotype	*Andaman*	*Gulf*	*Total*
A1	*8*	*8*	*16*
A2	-	*1*	*1*
A3	-	*1*	*1*
B1	*10*	*15*	*25*
B3	*1*	-	*1*
B4	-	*1*	*1*
B5	-	*1*	*1*
B6	-	*3*	*3*
Total	*19*	*30*	*49*

Divergence and geneflow

Low genetic divergence between the nesting green turtle populations of the Gulf of Thailand and the Andaman Sea was detected in both haplotypic (Gst=0.00311) and nucleotide levels (Nst=0.02838) as well as genetic distance (DTN=0.016±0.003). The estimated female mediated gene flows (Nm) from haplotype and nucleotide data were 161 and 17. This finding implies a lack of population subdivision between the nesting populations of the Andaman Sea and the Gulf of Thailand, and a sufficient degree of gene flow to prevent genetic differentiation between the two populations. The chi-square tests of genetic divergence of both Gst and Nst, revealed no significant differentiation (P>0.05) between the nesting populations of the Gulf of Thailand and the Andaman Sea.

DISCUSSION

Our results indicate that the two nesting green turtle populations from the Gulf of Thailand and the Andaman Sea were well mixed. The present geological boundary (the part of the Southern continent from Thailand to Malaysia peninsula down to Indonesia) seems not to effectively prevent the geneflow between the two populations as observed in invertebrate species (Kittiwattanawong, 1999; Supungul *et al.,* 2000; Kittiwattanawong *et al.,* 2001; Klinbunga *et al.,* 2001 Benzie, 1999; Bussarawit, 2003). However, this finding is not accord with the previous results of satellite tracking that the two nesting populations possessed separated feeding grounds and hence, they may be separated populations (Kittiwattanawong *et al.,* 2003). This contradiction leads us to discuss which of the two findings is the better understanding of the population structure.

First, genetic information may not echo the real time structure due to the high genome conservation, while tracking results reveal a present distribution of green turtle nesting populations. Extremely low genetic divergence rate in sea turtles has been reported in various genetic material levels such as protein (hybridization test, Karl *et al.,* 1995), chromosome (banding pattern, Bickham, 1981), Single-copy nuclear DNAs (Karl *et al.,* 1992), and microsatellite loci (Fitzsimmons et al., 1995). In addition, mtDNA evolution in turtles proceeds at a several-fold lower rate than "conventional" vertebrate pace (Avise et al., 1992; Bowen *et al.,* 1996). Such evidences suggest that a large part of genetic information has been remaining the same since the founding of the two populations from a common ancestor.

A limitation of our study is, however, the small sample size of the tracking. Only 11(Khram Island) and 9 (Huyong Island) turtles were tracked although we believe the sample size was appropriate considering the nesting population size at Khram Island (<100 nesters per year, Monanunsap and Charuchinda, 1994) and Huyong Island (12 individuals per year). In addition, the period of tracking period may be too short (9-126 days, Kittiwattanawong *et al.,* 2003) compared to the life span of the sea turtles (60 years, Seminoff, 2002). This suggests that the tracked turtles might do not stay at the same feeding ground, but wander to the wider range than we expect. Incorporate of this factor with a long range migratory ability may break down the barrier and hence leading to a mixing of the populations. Lastly, the sea level fluctuation may support the genetic-based finding. Geneflow between the two populations can occur by migration across seaways (via stepping stone mechanisms along nesting and feeding grounds or directly via long migratory pattern) in-betweens Malaysia peninsula-Sumatra, Sumatra-Java. These seaways have been closed and widen up over the time scale due to sea level fluctuation (Geyh *et al.,* 1979). Figure 3a shows that there were two periods that sea level were higher than the present during the past 140,000 years (Potts, 1983). High sea level would widen the Strait of Malacca, seaways in between Sumatra-Java, and consequently allowed higher geneflow of these two populations (Fig. 3c). In contrast, lower sea level would narrow the seaways or even closed them (Fig. 3b).

The present high sea level which started about 4,000 years ago may maximize the geneflow and cause a low genetic divergence (Fig. 2b, c, and d). This rise and fall of sea level may make two populations one non-differentiated population.

Table 2. Haplotype diversity (*h*), Nucleotide diversity (π), number of polymorphic nucleotide, and average number of nucleotide difference for the green turtle nesting populations from the Andaman Sea and the Gulf of Thailand. Diversity indices were calculated by DnaSp ver.3.99.5 (Rozas and Rozas 1999) and MEGA ver 2.1 (Kumar et al. 2001).

	Andaman	Gulf	Overall
Haplotypes diversity (*h*)	0.573 ±0.014	0.678±0.016	0.640±0.011
Nucleotide diversity (π)	0.294±0.068	0.264±0.008	0.272±0.005
No of polymorphic nucleotide	251	254	254
Average No of nucleotide difference	129	116	119

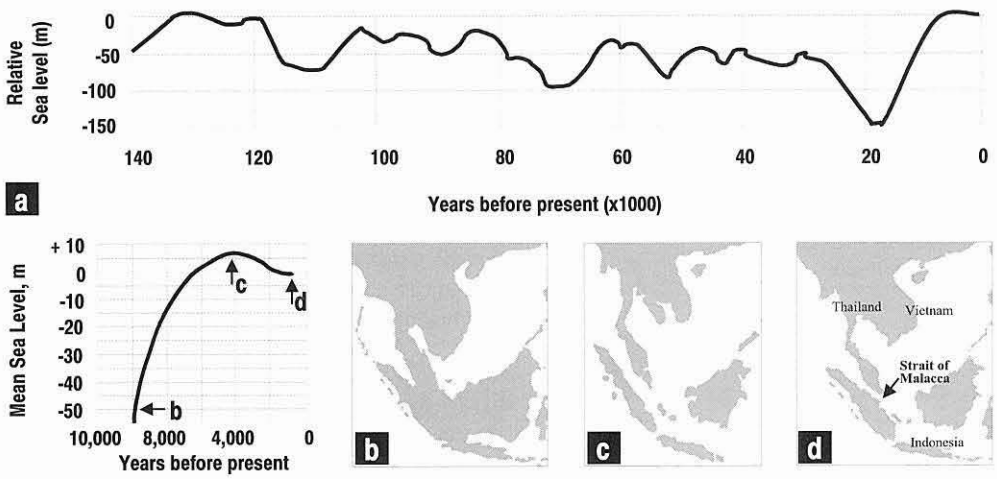

Fig. 3. a) Sea level fluctuation over the past 140,000 years ago (Potts 1983). b), c) and d) Sea levels and topographies of SPouth East Asia during 8000, 4000 years ago and at present, respectively (modified from Lekagul and McNeely 1977; Geyh et al. 1979).

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