

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

Kongkiat Kittiwattanawong¹⁾, Somchai Mananasup²⁾, Masato Kinoshita³⁾ & Kouji Nakayama⁴⁾

1) Phuket Marine Biological Center, Phuket 83000, Thailand. E-mail: kongkiat_k@hotmail.com

2) Sea Turtle Conservation Station, Department of Fisheries, Mannai Island, Rayong 21190 Thailand. E-mail: mannai@loxinfo.co.th

3) Department of Fisheries, Faculty of agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8520, Japan. E-mail: kinoshit@kais.kyoto-u.ac.jp

4) Laboratory of Marine Stock-enhancement Biology, Division of Applied Biosciences, Graduate School of Agriculture, Kyoto 606-8502, Japan. E-mail: nakayama@kais.kais.kyoto-u.ac.jp

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 AA ACT 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 ($G_{st}=0.00311$) and nucleotide levels ($N_{st}=0.02838$) as well as genetic distance ($D_{TN}=0.016\pm 0.003$). 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 (Figure 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, Sattapumin 2001). At the larger scale, this geological barrier may serve as a door to separate marine organisms between Indian and Pacific oceans.

A green turtle 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. Recently, satellite transmitted tracking of the nesting green turtle populations from the Andaman Sea and the Gulf of Thailand (Chapter 3 in this volume) suggested contemporary allopathic life cycles (Figure 1). However, such a only reflects the present scenario. An improved understanding may be further obtained by research on population genetic structure. Several kinds of genetic materials vary from proteins to nucleic acids can be employed (Avisé 1994). Since, genetic materials are inherited from one generation to another, information obtained from research on these genetic materials reflects from the summary of natural history the past till present (Futuyma 1986; Page & Holmes 1998).

This study was 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 & Greenberg 1983). 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 are revealed (Figure 1).

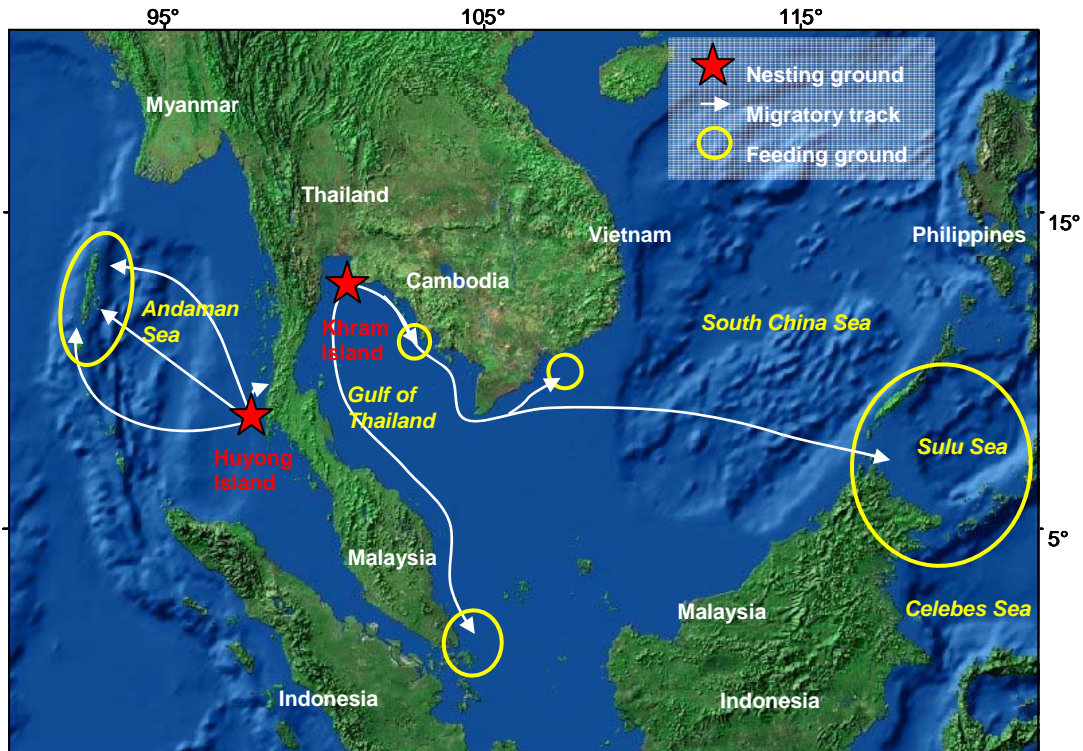


Figure 1. The two major nesting grounds of green turtles *Chelonia mydas* in Thailand (Khram Island in the Gulf of Thailand and Huyong Island in the Andaman Sea) with the satellite transmitted results showing the simplified migratory routes and their feeding grounds.

MATERIALS AND METHODS

The tissues

The samples were collected with supports from the Royal Thai Navy during 2001-2002. Generally, the staffs patrolled the beaches at night during high tide. After a turtle had laid eggs, the staffs scanned for a microchip tag at the both flippers (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 cm²) 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 medicines such as Gentian Violet, Povidiodine, or tetracycline oilment were placed to the wounds before releasing of the green turtles. 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 brought to analyze at Graduate School of Agriculture, Kyoto University. All the samples were exported under the permission of the CITES.

DNA analysis protocol

The detail analysis protocol of the control region mtDNA is shown in Appendix 1. In brief, 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 (Figure 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.

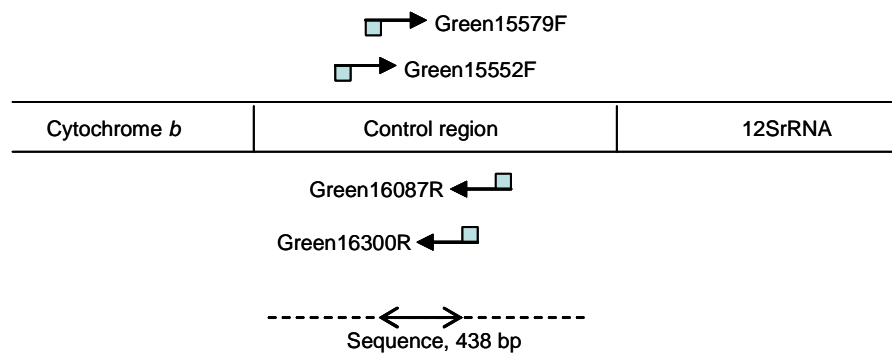


Figure 2. Location of the primers employed in the study overlaid to a none-scale mtDNA of *Chelonia mydas*. Arrows indicate the nucleotide synthesis directions. The line with both ends arrows indicates proximal length of nucleotide sequence (438 bp).

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 (Nei 1987) were calculated. Divergences between the two populations were calculated as G_{st} (based on haplotype frequencies, Hudson *et al.* 1992), N_{st} (based on nucleotide sequences, Lynch & Crease 1990), and Tamura-Nei's genetic distance (D_{TN} , Tamura & Nei 1993). N_{st} is similar to Fixation indices (F_{st}) described in Weir & Cockerham 1984. The difference is that N_{st} uses the Jukes & Cantor 1969 correction. Additionally, the differences in haplotype frequency among populations and the nucleotide divergence among haplotypes are both taken into account in the calculation of N_{st} (Ramey II 1995). G_{st} and N_{st} values range from 0 to 1 which indicate 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 test for any significant genetic differentiation between populations. Gene flow (N_m) between the two populations was estimated from N_{st} and G_{st} values using the formula $N_m = 0.5(1/N_{st} \text{ or } G_{st} - 1)$ (Wright 1951). N_m can be interpreted as the absolute number of individuals exchanged between populations per generation (Avice 1994). All calculations were performed using the program DnaSP version 3.99.5 (Rozas & 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 & 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) compared to the ones from the Andaman Sea (3 haplotypes *i.e.* A1, B1, and B3). The haplotype A2, A3, B4, B5, and B6 tended to endemic to the Gulf of Thailand, while there was only B3 that tended to restrict to the Andaman Sea.

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

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). 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 compared to the one in the Gulf of Thailand. However, nucleotide and haplotype

diversities in all cases might be slightly less than the actual value due to the calculations excluded gaps in the aligned sequences.

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	0.687±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

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 ($G_{st}=0.00311$) and nucleotide levels ($N_{st}=0.02838$) as well as genetic distance ($D_{TN}=0.016±0.003$). The estimated female mediated gene flows (N_m) from haplotype and nucleotide data were 161 and 17, respectively. This implied 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 G_{st} and N_{st} , revealed no significant differentiation ($P>0.05$) between the nesting populations of the Gulf of Thailand and the Andaman Sea.

DISCUSSION

Genetic information obtained from this study pointed out that the two nesting green turtle populations between the Gulf of Thailand and the Andaman Sea was 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). Moreover, this finding was not in agreement with the satellite transmitted tracking results reported in the Chapter 3 that the two nesting populations possessed separated feeding grounds and hence, leading to separated populations. Contradiction between the two findings leads to discussion of two schools of thought *i.e.* (1) tracking trusted postulation and (2) genetic trusted postulation.

In the first case, tracking results reveal a present distribution of green turtle nesting populations, while genetic information does not echo the real time structure due to the high genome conservation. Extremely low genetic divergence rate in sea turtles have 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 (Awise *et al.* 1992; Bowen *et al.* 1996). Such evidences suggested that a large part of genetic information has been remaining from the past since the founding of the two populations from a common ancestor.

Genetic trusted postulation may argue about the small sample size of the tracking study. Even though, the tracking result was retrieved from only 11 and 9 nesting green turtles from the Khram Island and Huyong Island, respectively, such numbers revealed a good representative compared to the number of yearly nesting green turtles at Khram Island (<100 nesters per year, Monanunsap & Charuchinda 1994) and Huyong Island (12 individuals per year). It can be claimed that the period of tracking period is relatively too short (9-126 days, chapter 3) compared to the life span of the sea turtles (60 years, Seminoff 2002). The tracked turtles might have not stayed at the same feeding ground all the time, but wandering to the wider expected rookeries. Incorporate of this factor with

long range migratory ability may break down the barrier and hence leading to a mixing of the populations. Lastly, the evidence from the sea level fluctuation tends to support the genetic-based finding. Gene flow between the two populations is likely to occur by migration across seaways (via stepping stone mechanisms along nesting and feeding grounds existed in the region or directly via long migratory pattern) in-between 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 2a showed that within 140,000 years ago (Potts 1983), there were two periods that sea level were higher than the one at present. These periods with high sea level affected the topography of the Southeast Asia by widening the Strait of Malacca, seaways in between Sumatra-Java, and consequently allowing higher gene flow of the both nesting turtle populations (Figure 2c). In contrast, the periods with lower sea level would narrow the seaways or even closed them as Figure 2b. Since the gene flow was maximized (hence, create low genetic divergence) in the period with higher sea level since 4,000 years ago and continue till present (Figure 2b, c, and d). This allows sea turtles to disperse back and forth between the two seas making both populations to have essentially become one non-differentiated population.

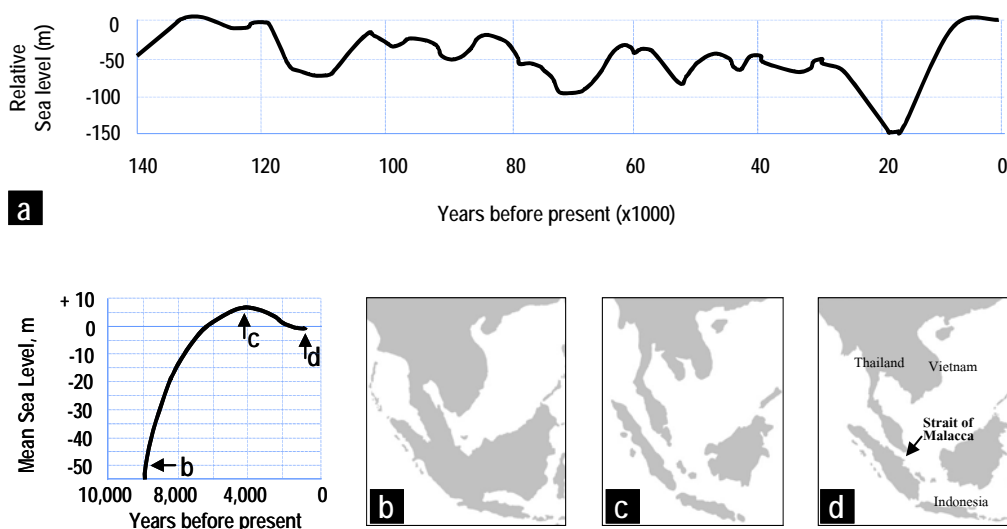


Figure 2. a) Sea level fluctuation over the past 140,000 years ago (Potts 1983).
b), c) and d) Sea levels and topographies of South East Asia during

8000, 4000 years ago and at present, respectively (modified from Lekagul & McNeely 1977; Geyh *et al.* 1979).

REFERENCES

- Aquedro, C.F., and Greenberg, B.D. 1983. Human mitochondrial DNA variation and evolution: analysis of nucleotide sequences from seven individuals. *Genetics* 103: 287-312
- Avise 1994. *Molecular markers, natural history, and evolution*. Chapman & Hall, New York. 511 pages.
- Avise, J.C., Bowen, B.W., Lamb, T., Meylan, A.B., and Bermingham, E. 1992. Mitochondrial DNA Evolution at a Turtle's Pace Evidence for Low Genetic Variability and Reduced Microevolutionary Rate in the Testudines. *Molecular Biology and Evolution* 9: (3) 457-473
- Benzie, J.A.H. 1999. Major genetic differences between crown-of-thorns starfish (*Acanthaster planci*) populations in the Indian and Pacific Oceans. *Evolution* 53: (6) 1782-1795
- Bickham, J.W. 1981. Two-hundred-million-year-old chromosomes: deceleration of the rate of karyotypic evolution in turtles. *Science* 212: 1291-1293
- Bowen, B.W., Bass, A.L., Garcia, R.A., Diez, C.E., Van, D.R., Bolten, A., Bjorndal, K.A., Miyamoto, M.M., and Ferl, R.J. 1996. Origin of hawksbill turtles in a Caribbean feeding area as indicated by genetic markers. *Ecological Applications* 6: (2) 566-572
- Fitzsimmons, N.N., Moritz, C., and Moore, S.S. 1995. Conservation and Dynamics of Microsatellite Loci over 300 Million Years of Marine Turtle Evolution. *Molecular Biology and Evolution* 12: (3) 432-440
- Futuyma, D.J. 1986. *Evolutionary biology*. Sinauer Associates, Inc, Massachusetts. 600 pages.

- Geyh, M.A., Kudrass, H.R., and Streif, H. 1979. Sea level changes during the late Pleistocene and Holocene in the Strait of Malaca. *Nature* 278: (5703) 441-443
- Hudson, R.R., Slatkin, M., and Maddison, W.P. 1992. Estimation of levels of gene flow from DNA sequence data. *Genetics* 132: 583-589
- Jukes, T.H., and Cantor, C.R. 1969. Evolution of protein molecules. In: Munro, HN (ed) *Mammalian protein metabolism*. Academic Press, New York, pp 21–132
- Karl, S.A., Bowen, B.W., and Avise, J.C. 1992. Global Population Genetic Structure and Male-mediated Gene Flow in the Green Turtle *Chelonia mydas* Rflp Analyses of Anonymous Nuclear Loci. *Genetics* 131: (1) 163-173
- Karl, S.A., Bowen, B.W., and Avise, J.C. 1995. Hybridization among the ancient mariners: characterization of marine turtle hybrids with molecular genetic assays. *Journal of Heredity* 86: 262-268
- Kittiwattanawong, K. 1999. Genetic structure of giant clam, *Tridacna maxima* in the Andaman Sea, Thailand. *Phuket Marine Biological Center Special Publication* 17: (1) 109-114
- Kittiwattanawong, K., Nugranad, J., and Tuernjai 2001. High genetic divergence between *Tridacna squamosa* living in the west and east coasts of Thailand. *Phuket Marine Biological Center Special Publication* 22:
- Klinbunga, S., Siludjai, D., Wudthijinda, W., Tassanakajon, A., Jarayabhand, P., and Menasveta, P. 2001. Genetic heterogeneity of the giant tiger shrimp (*Penaeus monodon*) in Thailand revealed by RAPD and mitochondrial DNA RFLP analyses. *Marine Biotechnology* 3: (4) 428-438
- Kumar, S., Tamura, K., Jakobsen, I.B., and Nei, M. 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics* 17: (12) 1244-1245
- Lekagul, B., and McNeely, J.A. 1977. *Mammals of Thailand*. Association for the Conservation of Wildlife, Bangkok. 758 pages.

- Lynch, M., and Crease, T.J. 1990. The analysis of population survey data on DNA sequence variation. *Molecular Biology and Evolution* 7: (4) 377-394
- Monanunsap, S., and Charuchinda, M. 1994. Nesting of sea turtles at Khram Island, Chonburi during 1988-1993 Fisheries Seminar. Department of Fisheries, Bangkok, pp 413-420
- Nei, M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York. pages.
- Nei, M., and Kumar, S. 2000. *Molecular Evolution and Phylogenetics*. Oxford University Press, New York. pages.
- Norman, J.A., Moritz, C., and Limpus, C.J. 1994. Mitochondrial DNA control region polymorphisms: Genetic markers for ecological studies of marine turtles. *Molecular Ecology* 3: (4) 363-373
- Page, R.D.M., and Holmes, E.C. 1998. *Molecular evolution: A phylogenetic approach*. Blackwell Science, Ltd, London. 346 pages.
- Phasuk, B. 1992. Biology and reproduction of green turtle *Chelonia mydas* in Thailand. *Thai Fisheries Gazette* 45: (1) 603-650
- Potts, D.C. 1983. Evolutionary disequilibrium among Indo-Pacific corals. *Bulletin of Marine Science* 33: (3) 619-632
- Ramey II, R.R. 1995. Mitochondrial DNA variation, population structure, and evolution of mountain sheep in the south-western United States and Mexico. *Molecular Ecology* 4: 429-439
- Rozas, J., and Rozas, R. 1999. DnaSP version 3: An integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* 15: 174-175
- Sambrook, J., Russell, D.W., and Sambrook, J. 2001. *Molecular cloning: A laboratory manual*, Third edition. Cold Spring Harbor Laboratory 999 pages.
- Seminoff, J.A. 2002. Global status of the green turtle (*Chelonia mydas*): A summary of the 2001 status assessment for the IUCN Red List Programme. In: Kinan, I (ed) *Proceedings of the Western Pacific Sea Turtle Cooperative Research and Management workshop*.

- Honolulu, HI: Western Pacific Regional Fishery Management Council, Hawaii, pp 197-211
- Supungul, P., Sootanan, P., Klinbunga, S., Kamonrat, W., Jarayabhand, P., and Tassanakajon, A. 2000. Microsatellite polymorphism and the population structure of the black tiger shrimp (*Penaeus monodon*) in Thailand. *Marine Biotechnology* 2: (4) 339-347
- Tamura, K., and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10: 512-526
- Thompson, J.D., Higgins, D.G., and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673-4680
- Weir, B.S., and Cockerham, C.C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* 38: 1358-1370
- Wright, S. 1951. The genetical structure of populations. *Ann. Eugenics* 15: 323-354