國立臺灣海洋大學

海洋生物研究所

碩士學位論文

指導教授: 程一駿 博士

西北太平洋綠蠵龜族群間的遺傳基因結構之研究

Investigating Population Genetic Structure among Green Turtle (*Chelonia mydas*) Rookeries of the Northwest Pacific Region

研究生: Peter Kowalski

中華民國 104 年 1 月

西北太平洋綠蠵龜族群間的遺傳基因結構之研究

Investigating Population Genetic Structure among Green Turtle (*Chelonia mydas*) Rookeries of the Northwest Pacific Region

研究生: 高彼德　　Student: Peter Kowalski

指導教授: 程一駿　　Advisor: I-Jiunn Cheng

國立臺灣海洋大學

海洋生物研究所

碩士學位論文

A Thesis
Submitted to the Institute of Marine Biology
College of Life Sciences
National Taiwan Ocean University
in partial fulfillment of the requirements
for the degree of
Master of Science
in
Marine Biology
January 2015
Keelung, Taiwan, Republic of China

中華民國 104 年 1 月

Acknowledgements

I would like to first thank my parents, as well as my sister and brothers for their unwavering love and support, which has helped guide me throughout the years. Also thanks to May for always being loving, caring, supportive, and understanding. I'm grateful for having you all in my life.

Thanks to National Taiwan Ocean University, the Office of International Affairs, and our university president, Professor Ching-Fong Chang, who have all supported and enrich my time at NTOU through scholarships and help whenever needed.

A special note of appreciation to my supervisor and mentor, Professor I-Jiunn Cheng: Thank you for taking me under your wing and providing me with the opportunity to work with you on this exciting project. I have learned a great deal about conservation, biology and life from you. Your guidance, expertise, support and sense of humour have made my time with your lab a truly unforgettable learning experience. I will miss your weekly Far Side comics, and I wish you and your lovely family the best in the years to come.

I would also like to thank my lab mates, as they have always been incredibly helpful in maneuvering my life as a student here. A special thanks to Tseng-Cheng Tsung, who has been a big brother to me here.

Our research could not be conducted without the help of dozens of volunteers every year at Wangan, Lanyu and Xiaoliuqiu. The same goes for the local patrolmen in Wangan: Tsui-Bei, Guan-Da and Yong-Xiang, as well as Jojo Kao and Pei-Yu Lin, all of which I'm proud to call my friends. Thank you all for your hard work and dedication to understanding and preserving Taiwan's green turtles – they need all the help they can get.

Lastly, I would like to thank the countless wonderful people I've met in Taiwan. It is the friendliest country I've ever seen, and that is something to take pride in - 台灣加油!

摘要

綠蠵龜(*Chelonia mydas*)，是種長壽且廣泛分布於各大洋的海洋爬行動物，因過度被人為捕殺等因素已被 IUCN 列入瀕危物種的紅色名錄當中。台灣與日本分別都是西北太平洋綠蠵龜的重要生活棲地，此外台灣的望安、蘭嶼與小琉球及日本的西表島與石垣島分別也都是西北太平洋綠蠵龜的重要產卵地，因此在此區域內，綠蠵龜受到高度的法令保護與重視。本論文的研究目的為 a)提供西北太平洋綠蠵龜族群結構資訊 b)了解西北太平洋的綠蠵龜族群群落數量 c)評估雄性綠蠵龜所引發族群間基因交流的程度。利用 6 組標準化的多類型微衛星基因座分析在 2003 年及 2013 年於台灣及日本共 5 個不同產卵地所採集到共 140 隻西北太平洋的產卵綠蠵龜序列，結果顯示並沒有明顯的族群差異存在(F_{ST}:0.00634~0.06493)，此外不同群體間的基因有高度交流的情況發生(*Nm*:3.6~39.17)，而利用 STRUCTURE 分析的結果呈現出 K=1，這代表望安，蘭嶼、小琉球、西表島與石垣島的產卵綠蠵龜為同一個逢機交配族群，此結果與利用粒線體核酸所分析出的結果全然不同。若以靠母系遺傳的粒線體核酸分析西北太平洋區域的產卵母龜，其結果顯示有數個不同的綠蠵龜族群生活於此，但本研究以細胞核核酸進行分析，結果顯示西北太平洋僅有 1 個族群存在，此結果顯示雄性海龜在不同地區間綠蠵龜的基因交流佔有很重要的角色。

關鍵字: *Chelonia mydas*, 族群結構, 微衛星, 台灣, 日本, 基因流

Abstract

The green turtle (Chelonia mydas) is a long-lived, wide-ranging and endangered marine reptile. Important green turtle rookeries of the northwest Pacific region include Wangan, Lanyu, and Xiaoliuqiu in Taiwan, and Iriomote and Ishigaki in Japan.140 genetic samples from nesting females in this region were collected between 2003 and 2013. The purpose of this study was to a) provide information on population structure, b) determine the number of genetic populations, c) assess the potential for male-mediated gene flow among rookeries. The results of 6 polymorphic microsatellite loci were standardized between three laboratories. Over all loci, values of population differentiation (F_{ST}:0.00634~0.06493) were very low, exhibiting a high rate of gene flow (*Nm*:3.6~39.17) among rookeries of this region, which sharply contrasts past studies using mtDNA. These results were supported by a K=1 result from population assignment tests in the program STRUCTURE, indicating one panmictic genetic population among these 5 rookeries. Very high differentiation at mtDNA compared to microsatellite markers suggests the potential for male-mediated gene flow, which should be tested directly by sequencing mtDNA control region for this dataset.

Key words: *Chelonia mydas*, population structure, microsatellites, Taiwan, Japan, gene flow

Table of Contents

List of Figures

List of Tables

List of Tables (continued)

General Introduction

Sea turtles are reptiles (class: Reptilia) of the order Testudines (also known as Chelonii), which encompasses both freshwater and marine turtles, as well as tortoises. The superfamily Chelonioidea contains all seven extant species of sea turtle, which began to diverge from their ancestral form approximately 150 MYA. Most species of sea turtle share similar life history characteristics, with some variation among species and populations. Generally speaking however, sea turtles are long-lived animals which grow and mature slowly, occupying multiple habitats throughout their life cycle, including neritic, pelagic, estuarine and beach habitats (Seminoff 2004). Sea turtles have a circumglobal distribution and can be found in tropical, sub-tropical and temperate waters (Seminoff 2004). One key characteristic of sea turtle species is their ability to migrate great distances, with some species even crossing ocean basins from foraging grounds to reach their rookeries of origin through a behaviour known as natal philopatry (Bowen et al., 1995).

Sea turtles are an ancient and hardy group of species, having survived massive extinction events as well as glaciations. However, within an average human lifespan, many sea turtle populations have come close to the brink of extinction. Historical and ongoing anthropogenic effects such as hunting for meat and jewelry, increased by-catch from expanding global fisheries, coastal and marine habitat degradation, ingestion of plastic waste, and entanglement in various forms of marine pollution (discarded fishing lines, nets, etc.) has reduced population sizes to the point where many sea turtle species are at risk of extinction (Seminoff 2004; Lewison and Crowder 2007). These factors, in combination with a late age at maturity, make for slow and difficult population recovery (Crouse et al., 1987).

Introduction

Study Species: Green Turtle (*Chelonia mydas*) (Linnaeus, 1758)

Taxonomy and Physical Description

The green turtle (*Chelonia mydas*) has the following taxonomic placement:

Kingdom: Animalia
Phylum: Chordata
Class: Reptilia
Order: Testudines
Family: Cheloniidae
Genus: *Chelonia*
Species: *mydas*

The green turtle is the largest turtle in both size and weight of the family Cheloniidae, averaging about 120cm straight carapace length and approximately 68-190 kg in size at adulthood. Next to the leatherback, the green turtle is the second largest extant sea turtle (Pritchard and Mortimer 1999). The green turtle gets its common name from the colour of its fat, which is green due to its predominantly herbivorous diet of seagrass and algae. Green turtles can be identified by the following external physical characteristics: smooth oval shell, 5 vertebral scutes, 4 pairs of costal scutes, 4 inframarginal scutes, a pair of prefrontal scales and 4 pairs of postorbital scales on the head, as well as a serrated lower jaw (Fig. 1), though occasionally physical mutations can occur (Ergene et al., 2011).

Conservation Status in Taiwan

The green turtle is the only species of sea turtle known to nest in Taiwan. Major rookeries (nesting areas) include the islands of Wangan, Lanyu, and Xiaoliuqiu, which are just off-shore from the main island of Taiwan. Other rookeries include DongSha and Taiping

islands, which are conisderably further from the main island of Taiwan, located in the South China Sea (Cheng 1996; Cheng 2008; Cheng 2009).

Since 1989, the green turtle has been listed as Category I in the Wildlife Protection Act of the Republic of China (Taiwan), meaning it is listed as an endangered species and is protected by from being killed or harassed. It is also listed as Endangered by the IUCN and listed as Appendix I by the Convention on Trade in Endangered species (CITES), meaning that this species is threatened with extinction, and international trade in specimens is prohibited unless the purpose of import is not commercial, though CITES permits can be granted for scientific purposes (Seminoff 2004).

Major threats to green turtles in Taiwan include direct harvest, egg poaching, fisheries bycatch, sand mining and marine pollution (Cheng et al., 2009). Evidence has shown that green turtles nesting in Taiwan migrate into the territorial waters of the Taiwan, Japan, The Phillippinnes, and mainland China (Cheng et al. 2000). This type of multi-national habitat use has been seen in many populations of green turtles, and implies that one country cannot be solely responsible for the effective management of green turtle populations. Therefore, it is critical to study the life history, habitat use and mating patterns of green turtle populations in order to suggest potential management cooperation between nations that share responsibility for this highly mobile endangered species (Cheng et. al., 2008; Nishizawa et al., 2011).

Distribution, Life History and Mating System

After green turtle eggs are laid by adult females under the beach sand, they incubate for approximately 50-60 days. When the juveniles emerge, they will typically move immediately to the ocean. Then, they immediately begin swimming rapidly through open water. After this, it is difficult to say with any certainty what happens in every population of green turtles, as juveniles are rarely encountered in the wild. It is suggested that they initially settle under floating mats of vegetation, being carried by ocean currents for an unknown number of years (Carr and Meylan 1980, Musick and Limpus, 1997). After

reaching approximately 20-40cm in curved carapace length, they will disperse into neritic habitats, predominantly occupying the benthic zone (Balazs, 1982; Jensen et al., 2013).

Estimates of mean age at maturity tend to vary between populations and the method used to make the estimate, though recent findings from a Pacific population of green turtles (in Hawaii) has recently estimated age at first maturity being 23 years of age (Van Houtan et al., 2014).

Upon reaching sexual maturity, female green turtles will migrate from foraging grounds to breeding every few years. The migrations between foraging and nesting grounds can vary from hundreds to thousands of kilometers, depending on the population and individual (Limpus 2007). However, the movements of adult male green turtles are not well-studied overall.

A growing number of studies have suggested that males may mediate gene flow between populations by mating opportunistically between genetically distinct nesting populations (Roden et al., 2013, Nishizawa et al., 2011). This type of mating can occur in the three major habitats utilized by adult sea turtles: nesting areas, mixed foraging aggregations and migratory routes. During the nesting season, females and males aggregate offshore from nesting areas. There, females can mate with either males of the same natal origin, or potentially with males of a geographically distant origin that have traveled in search of mating opportunities. This “mate-seeking behavior” has been reported in males that have migrated between several beaches throughout a nesting season, purportedly to mate with females residing offshore (Wright et al., 2012).

After the nesting season, females migrate to foraging areas that may have individuals of various natal origins, known as “mixed foraging aggregations”. These areas also provide an opportunity for opportunistic mating between turtles sourced from different rookeries (Bass et al., 2006). Lastly, females undertaking migrations may encounter males along their migratory route, which may also result in opportunistic mating and the flow of genetic material between geographically and demographically distinct populations

(Bowen and Karl 2007 and Wright et al., 2012).

Sex Bias in Population Research

Since green turtles exhibit precise natal homing to locate a particular nesting area, females will often return to the same beach to lay eggs. Females leave a set of large, conspicuous tracks on the sand when ascending a beach, so they can be located and studied by researchers with relative ease (Wang and Cheng 1999). This has led to patterns of female migration to be very well-studied, since females can easily be tagged with metal tags that contain unique ID numbers which can be used in a mark-recapture method to track their movements and return to natal beaches. They can also be equipped with satellite transmitters, which can track their habitat use and post-nesting migrations.

In contrast, male green turtles in most populations typically only come ashore when they are sick, injured or dead; with the exception of Hawaiian green turtles, which exhibit basking behaviour (Karl 1992; Whittow and Balazs 1982). Therefore, male turtles must be located and either studied underwater or captured, which is fraught with difficulty. There is a paucity of data from tag returns of male turtles, and even less research using satellite tracking (Luschi, et al. 2003). This sex-based differential in difficulty of data collection has led to an overwhelming majority of research being conducted on female sea turtles during and after nesting, and on their offspring during incubation and directly after their emergence from a nest.

Genetic Studies of Population Structure

Determining the genetic structure of sea turtle populations is an important step in determining and understanding the interactions occurring between green turtle populations at a local, regional and global level (Nishizawa et al., 2011; Roden et al. 2013). Although populations may be geographically distinct in terms of nesting philopatry, green turtles can undertake both short and long distance migrations between

nesting and foraging sites, which often involves crossing through international jurisdictions (Hirth 1997). This is why determining whether mating is occurring between individuals of distinct rookeries, nations and regions is an important step in appropriately managing green turtle stocks (Nishizawa et. al., 2011; Cheng et al., 2000).

Population genetic studies of green sea turtles utilizing mitochondrial DNA (mtDNA) have supported evidence of natal philopatry from mark-recapture tagging studies. Mitochondrial haplotypes are inherited from a mother to her offspring, and due natal philopatry, as well as processes such as colonization, founder effect and genetic drift, certain haplotypes will become fixed in a nesting population (Karl et al., 2012). While mtDNA studies may be useful for determining maternal philopatry and maternal population subdivision, mtDNA data cannot reliably determine ongoing gene flow between rookeries mediated by males (Karl 1992; Bowen 1992).

Microsatellites (also known as Short Tandem Repeats, or STRs) are short portions of a DNA sequence where a motif of one to six bases is repeated in tandem. Microsatellites occur in very high frequency in the genome of virtually any species and have extremely high variability. These powerful nuclear markers are co-dominant and follow Mendelian inheritence. Flanked by conserved regions of DNA, primers can be designed for species to excise targeted microsatellite loci for amplification and analysis (Selkoe and Toonen 2006) (Figure 2).

Due to their bi-parentally inherited nature, microsatellite markers have proven to be useful in providing a more comprehensive view of gene-flow (Selkoe et al., 2006). Studies of genetic structure between green turtle rookeries worldwide have revealed that many populations sharing common feeding grounds are often homogeneous at nuclear loci, although they can differ in mtDNA haplotype frequencies (Karl 1992). Researchers have suggested that highly reduced population structuring (low genetic differentiation) at nuclear markers relative to mitochondrial markers can be explained by male-mediated gene flow (Karl 1992; Jensen 2004).

There is currently a paucity of information regarding the population genetic structuring of green turtles in the Northwest Pacific region. Taiwan has three island rookeries with long-term projects established: Wangan, Lanyu and Xiaoliuqiu. Past studies of the post-nesting migrations of satellite-tracked females at Wangan and Lanyu have provided evidence of the Ryukyu Archipelago of Southwestern Japan being an important migratory area for green turtles (Cheng et al 2000, Wang et al, 2014) (Figure 3). Studies in Japan have also identified the Ryukyu Archipelago as a nesting and foraging area for green turtles (Nishizawa 2013). As such, the evidence suggests that the green turtles of Taiwan and Southwestern Japan likely form a mixed foraging aggregation, where opportunistic mating, and subsequent gene flow, may be occuring. However, mtDNA evidence suggests that strong genetic differentiation exists between WangAn Island, Orchid Island, and the nearby Japanese islands of Iriomote and Ishigaki in Japan's Ryukyu Archipelago, all of which are less than 600km apart (Nishizawa et. al. 2011)(Figure 4). A similar, yet even stronger pattern of differentiation was seen when comparing mtDNA structuring between Wangan and Lanyu (Cheng et al 2008).

Purpose

Due to the limitations of mtDNA in detecting ongoing gene flow, a study of five green sea turtle rookeries of the northwest Pacific region using polymorphic microsatellite loci was proposed to:

a) provide information on the population structure,
b) determine the number of genetic populations,
c) assess the potential for male-mediated gene flow among rookeries

Materials and Methods

Study Site

This project is a collaboration between National Taiwan Ocean University, Kyoto University and the NOAA Southwest Fisheries Science Centre. Field work took place on 5 island rookeries (nesting sites); three in Taiwan and two in southwestern Japan (Figure 4). The Taiwanese sites are as follows: Wangan Island (23° 22'N, 119°30'E) is located in the Penghu archipelago, approximately 60 km from the southwest coast of Taiwan. It is approximately 7.4km² with 9 nesting beaches. Lanyu (22°-08'N, 121°50'-60 E) is located approximately 65 km from the southeast coast of Taiwan. It is approximately 46 km² with 3 nesting beaches. Xiaoliuqiu (22°20'N, 120°20'E) is located approximately 14km off the southwest coast of Taiwan. It is approximately 6.8 km² with 5 nesting beaches. The Japanese sites are as follows: Iriomote (24°17'33'N, 123°51'E) is located approximately 185 km from the northeast coast of Taiwan. It is approximately 289km² and has one nesting beach. Ishigaki (24°20'26'N, 124°9'20E) is located approximately 300 km from the northeast coast of Taiwan. It is approximately 229km² and has two nesting beaches.

Sample Collection

At Taiwanese sites, nesting female green turtles were sampled on beaches at Wangan, Lanyu and XiaoXiaoliuqiu islands between July and September during nightly patrols (Figure 4). Individual females could be identified by their unique ID numbers, from either coded metal tags (National Band & Tag Co.) which are installed on each of their hind flippers, or passive integrated transponder (PIT) tags (Avid Plc.), which are embedded under the skin of the hind flippers and read with a scanner. This way, an individual would only be sampled once. Samples were collected in Wangan between 2004 and 2013, in Lanyu between 2005 and 2013 and in XiaoXiaoliuqiu between 2011 and 2013. A small piece of tissue (~2mm in diameter) was removed from a hind flipper of each sampled female using sterile hole punch. Collected tissue samples were stored in dimethyl sulfoxide (DMSO solution).

At Japanese sites nesting female green turtles were sampled at Ishigaki and Iriomote islands between May and September during nightly patrols (Figure 4). Individual females could be identified by their unique tag ID numbers, using either metal tags (National Band & Tag Co.), custom plastic tags, or PIT tags. Samples were collected in Iriomote and Ishigaki islands between 2003 and 2010. A small piece of tissue (approximately 5mm in diameter) was excised from the hind flippers using a hole punch. Collected tissue samples were stored in 99% EtOH.

DNA Extraction

In Taiwan, DNA was extracted using the gSYNC DNA Extraction Kit (GeneAid Biotech Ltd.), following manufacturer protocol. The concentration of purified DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.). In Japan, DNA was extracted using the Blood & Tissue Genomic DNA Extraction Miniprep System (Viogene), following manufacturer protocol.

Microsatellite Genotyping

In order to determine the genetic characteristics of sampled green turtles, 6 microsatellite markers previously found to be variable in green turtles were utilized (Table 1). The markers A6, B108, D1, D2 and D115 were described in Dutton and Frey (2009), and marker D102 was described in Roden et al. (2013).

Polymerase Chain Reaction

Microsatellite loci were amplified using polymerase chain reaction (PCR) using negative controls (no template control), and examined for success using gel electrophoresis. In Taiwan, the reactions were 25-µL, following the protocol of Dutton and Frey (2009): each reaction contained 2 mM MgCl2, 0.6 mm each dNTP, 0.3 µm of each primer, 0.025

U/μL Taq DNA polymerase, and ~10 ng of template DNA. PCR was performed using a Veriti Thermal Cycler (Applied Biosystems). PCR conditions were as follows: initial denaturation (94 °C, 3 minutes) was followed by 35 cycles of denaturation (94 °C, 30 seconds), followed by annealing (55 °C, 30 seconds) and extension (72°C, 30 seconds). The final extension was at 72 °C for 5 minutes (Dutton and Frey 2009). In Taiwan, primers were forward-labelled using coloured fluorescent dyes from the DS-33 dye set (Applied Biosystems) (Table 1).

In Japan, loci were amplified using PCR with negative controls (no template control well), and tested using gel electrophoresis. The PCR protocol was as follows: 25-μL PCR reaction: 3.0-μL template,12.5-μL AmpliTaq Gold 360 Master Mix (Applied Biosystems), and 1.0-μL each primer (10 μM). Primers were forward-labelled using Beckman D4 fluorescent dye (Beckman Coulter Inc.) (Table 1). Loci were not multiplexed, so all samples and loci were run in independent wells.

Allele Scoring

In Taiwan, amplification products were separated by capillary electrophoresis on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). The outputs (visual peaks corresponding to fragment sizes) were analyzed using the GeneScan 600 LIZ Fluorescent Size Standard (Applied Biosystems), and raw data generated was imported into GeneMapper v5.0 software. Alleles were scored and exported into Microsoft Excel 2010 (Microsoft Corp.).

At Kyoto University, amplification products were seperated by capillary electrophoresis on a Beckman GenomeLab GeXP Genetic Analysis System (Beckman Coulter Inc.). The outputs (visual peaks corresponding to fragment sizes) were analyzed using the GenomeLab DNA Size Standard Kit 400 (Beckman Coulter Inc.). Raw data generated was imported into the Fragment Analysis Module, which is part of the Genomelab GeXP Genetic Analysis System (Beackman Coulter Inc.). Alleles were scored and exported into Microsoft Excel 2010 (Microsoft Corp.).

Dataset Standardization

Although microsatellite alleles are labelled according to their estimated size, when running the same sample on different machines, allele size shifts can occur. Therefore, the scoring of alleles may vary between labs (Gill et al., 1994; Ellis et al., 2011). In order to prevent this type of error, internal size standards in the form of 4 reference samples from Lanyu were included in runs at all labs to facilitate standardization of allele sizes. Locus-specific allele sizes, verified through multiple runs on different machines, were provided by the National Oceanic and Atmospheric Administration (NOAA) (Dr. Peter Dutton, NOAA, unpublished data). Using the internal reference samples, the NTOU and Kyoto U datasets were calibrated using the allele size designations of the NOAA standard by adjusting for size shift between datasets. This type of standardization method has achieved success for multinational collaborations in salmon research projects (Ellis et al., 2011).

Standardized data was subsequently changed into the correct format for CONVERT v1.31 software (Glaubitz 2004). CONVERT re-formats data files for compatibility with a variety of population genetic software formats.

Detection of Null Alleles and Error Checking

The software Micro-Checker v.2.3.3 (van Oosterhout et al., 2004) was utilized in order to determine the presence of null alleles. Null alleles are non-amplified alleles which may cause a false detection of a homozygous individual, which can negatively influence interpretation of allele frequencies (van Oosterhout et al., 2006). This is typically caused by mutations at a primer site (Dakin & Avise 2004). This software was also used to test for scoring error due to stutter. Evidence of large allele dropout (preferential amplification of smaller alleles (Wattier et al. 1998)) was also tested using Micro-checker. All analyses used 1000 iterations.

Calculation of Summary Statistics

The software Arlequin v3.5.1.3 (Rousset 2014) was used to calculate summary statistics for the six microsatellite loci in this study. This includes the number of alleles, expected heterozygosity, observed heterozygosity. The software Cervus 3.0.7 (Kalinowski et al. 2007) was used to calculate polymorphic information content (PIC), which is a measure of polymorphism that describes a locus's usefulness in analysis (Botstein et al. 1980).

Arlequin was used to calculate summary statistics for the 5 populations in this study, which included measures of expected and observed heterozygosity.

Evaluation of Hardy-Weinberg Equilibrium and Linkage Disequilibrium

The software GENEPOP v.4.2 was used to evaluate the probability of loci and populations conforming to Hardy-Weinberg Equilibrium (HWE) using Fisher's exact test with Markov Chain parameters of 1000 for dememorization number, 20 batches and 5000 iterations per batch.

The software ARLEQUIN v.3.5 (Excoffier and Lischer 2010) was used to test for linkage disequilibrium (LD) with 1000 permutations. LD refers to the random association of alleles between loci. Large populations exhibiting random mating without selection, should have close to no LD. LD is often regarded as evidence of population mixing and selection (Smit-McBride et al. 1988).If significant deviations from HWE and LD are found, this suggests that non-random mating, mutations, population mixing, and natural selection may be occurring in these populations (Smit-McBride et al. 1988).

Determining Population Differentiation and Gene Flow

The software ARLEQUIN v.3.5 was used to measure pairwise population differentiation (F_{ST}) and gene flow (*Nm*) values for each pair of populations (Weir and Cockerham 1984). F_{ST} (also known as fixation index) measures variation in allele frequencies among

subpopulations, providing a measure of population differentiation that ranges between 0 and 1. An F_{ST} value of zero would imply complete panmixis, where all subpopulations measured have identical allele frequencies. An F_{ST} value of 1 would imply complete reproductive isolation, where all subpopulations measured are fixed for completely different alleles. Hence, where a high level of gene flow occurs between subpopulations, F_{ST} values will be closer to zero. Conversely, where a low level of gene flow is occuring between subpopulations, F_{ST} values will be closer to 1. Gene flow (*Nm*) values, derived from F_{ST}, represent the estimated number of theoretical migrants between populations per generation (Weir and Cockerham 1984).

Population Assignment Tests

The software STRUCTURE v2.3.4 (Pritchard et al. 2000, Falush and Pritchard 2003) was used to produce an unbiased clustering of individuals into theoretical populations based on their genetic composition. This software utilizes a model-based Bayesian clustering method to infer population structure using genotype data independent of sampling location. This program can be used to demonstrate the presence of population structure, identify distinct genetic populations and assign individuals to populations. For computation, theoretical values of K (maximum number of genetic populations) are provided by the user, and individuals are assigned (based on probability) to a population, or to multiple populations if an individual's genotype indicates admixture. In order to estimate the true number of genetic populations (K) in this study, 5 independent runs of K = 1 to K= 10 were performed using 500 000 Markov Chain Monte Carlo repetitions, followed by a burn-in stage of 500 000 repetitions.

The results of STRUCTURE analysis were compiled in STRUCTURE Harvester v0.6.94 (Earl and von Holdt 2012), a web-based program which compiles STRUCTURE output files to determine the natural log of likelihood, Ln[Pr(X | K)], for each value of K. Where (Pr) is the probability, of data (X) at each theoretical number of genetic populations (K). The most probable true value of K is one that shows the highest reported likelihood value,

with increasing variance at successive values of K (Pritchard et al. 2000).

STRUCTURE Harvester was also utilized to create STRUCTURE data files which are compatible with the software CLUMPP v1.1.2 (Rosenberg and Jakobsson 2007). CLUMPP is used to permute the cluster output data from STRUCTURE (which is divided into independent runs) so that they match up as closely as possible. CLUMPP output files are then imported into the software DISTRUCT v1.1 (Rosenberg 2004). This program visualizes the genetic clusters identified by STRUCTURE in coloured plot format. These plots can be used to further interpret patterns of genetic population structure by visualizing the probability of each individual belonging to a number of theoretical genetic populations (K), which are represented by separate colours. If there is a high level of genetic structuring between populations, vertical clustering is visualized. If there is a high level of gene flow between populations, there will be no definitive assignment of individuals to particular clusters (ie. proportionate relationships exist to all tested population clusters among individuals) (Rosenberg 2004).

Assessment of Potential for Male-Mediated Gene Flow

Available F_{ST} data from mtDNA and microsatellites from Cheng et al. (2008) and Nishizawa et al. (2011) were compiled into a table to provide a rough assessment of potential for male-mediated gene flow, in order to determine suitability of analysis at mtDNA control region.

Results

Sample Collection

The total number of samples collected for analysis was 140. The sample sizes (n) ranged from as low as 8 individuals (Xiaoliuqiu) to as many as 49 individuals (Ishigaki) (Table 2). The average number of samples per site was 28 (±17.79 SE).

DNA Extraction & Polymerase Chain Reaction

DNA extraction was successful for all tissue samples. PCR amplification experienced variation in success (Table 3). Most loci had a high PCR amplification success rate of over 95%. Locus A6 had the highest amplification success (99.3%), while locus D115 had the lowest (91.4%). The overall PCR amplification success rate for this study was 96.53% (±2.78 SE). No individuals were removed from analysis due to failure of amplification at a locus.

Dataset Standardization

The reference samples run at the Kyoto University laboratory largely conformed to the locus-specific reference alleles provided by the NOAA, and were easily standardized. However, comparing the allele sizes of reference samples run in in Taiwan, there was evidence of locus-specific shifts of between 0.67 bp and 3.20 bp in size between loci. Prior to calibration, allele sizes for samples run in Taiwan were higher at all loci and reference samples. Among reference samples and runs, Locus A6 exhibited a 1.01 bp shift (±0.33 SE), Locus B108 exhibited a 2.39 bp shift (±0.06 SE), Locus D1 exhibited a 2.27 bp shift (±0.04 SE), Locus D2 exhibited a 2.3 bp shift (±0.07 SE), Locus D102 exhibited a 3.2 bp shift (+/-0.17 SE), and Locus D115 exhibited a 0.67 bp shift (±0.36 SE). The majority of allele sizes were calibrated by locus and successfully fitted to allele designations, and conformed to separation by single-unit sizes (e.g. 4bp for tetranucleotide loci). At locus D115, one individual from Lanyu and one from Ishigaki could not be scored using the allele designations due to an allele size that was squarely between two repeats. They were subsequently removed from the analysis at this locus.

Null Alleles and Error Checking

Results from MICRO-CHECKER did not indicate any evidence of null alleles throughout the data set. When testing for errors, two simple allele size input errors were detected. After correction, no scoring errors due to stutter were detected, nor was there any

evidence of large allele dropout in any population.

Summary Statistics, Tests for Hardy-Weinberg and Linkage Disequilibrium

Summary statistics for loci are listed on Table 4. Locus D115 had the highest number of alleles (21) and the highest PIC (0.907). Locus D102 had the lowest number of alleles (5) and the lowest PIC. The mean number of alleles over all loci was 12.167 (±5.981 SE). The mean PIC over all loci was 0.757 (±0.212 SE). Observed heterozygosity values ranged from 0.391 (Locus D102) to 0.914 (Locus A6), with a mean value of 0.689 (±0.215 SE) over all loci. Expected heterozygosity values ranged from 0.380 (Locus D102) to 0.917 (Locus D115), with a mean value of 0.780 (±0.2 SE) over all loci. Half of the six loci used in this study conformed to HWE (B108, D1 and D2), while the others did not ($p<0.05$).

Summary statistics by population are listed on Table 5. The lowest observed heterozygosity was in Xiaoliuqiu at 0.577 (±0.299 SE), while the highest was in Iriomote at 0.779 (±0.224 SE). The lowest expected heterozygosities were in Xiaoliuqiu, Lanyu, and Ishigaki at 0.742 (±0.120, 0.244 and 0.173 SE, respectively). Xiaoliuqiu had the lowest average number of alleles over all loci at 4.50 (±1.64 SE). Ishigaki had the highest average number of alleles over all loci (10.00 ±4.60 SE). Only one population was in HWE (20% of total sampling sites), while the remaining populations were not ($p<0.05$).

Significant linkage disequilibrium ($p<0.05$) was detected at all loci (Table 6). A6 and D2 showed the most significant linkage, exhibiting linkage with 100% of other loci. Loci B108 and D102 exhibited significant linkage with 80% of other loci. Locus D1 and Locus D115 exhibited the least amount of linkage, with significant linkage with 60% of other loci.

Population Differentiation and Gene Flow

Among all sampling sites, the mean F_{ST} value was 0.03985 (±0.01673 SE) (Table 7). The highest amount of differentiation was seen between Iriomote and Xiaoliuqiu (F_{ST} = 0.06493, $p<0.05$). The lowest amount of differentiation was seen between Iriomote and Ishigaki (F_{ST}=0.00634, p=0.06029), followed by Lanyu and Xiaoliuqiu (F_{ST}= 0.02407, p= 0.10553). The lowest amount of significant differentiation was seen between Iriomote and Lanyu (F_{ST}=0.03391, $p<0.05$).

Estimated gene flow (*Nm*) was highest between Iriomote and Ishigaki (39.17 migrants/generation) (Table 7). The lowest level of gene flow was between Iriomote and Xiaoliuqiu (3.60 migrants/generation).

Population Assignment Tests

From the software STRUCTURE, the highest mean log-likelihood value, L(K), with the least amount of variance was K=1, where K is the maximum number of genetic populations in the data set (Figure 5)

Based on visual analysis of the graphical output from CLUMPP, (using STRUCTURE output data) the most likely K value is K=1. At K=2, no distinct vertical clustering is seen between populations, and individual probabilities of membership to any theoretical K appears relatively even across populations. With increasing K values, the same pattern is seen, with approximately equal probabilities of most individuals belonging to any given number of populations (Figure 6)

Assessment of Potential for Male-Mediated Gene Flow

The comparison indicated that pairwise comparisons of F_{ST} values from mtDNA and 6 microsatellite loci invariably displayed much higher differentiation at mtDNA, exceeding differentiation at microsatellite loci by at least an order of magnitude in each pairwise

comparison, with the exception of Ishigaki and Iriomote, which were two orders of magnitude higher at mtDNA control region (Table 8).

Discussion

Genetic Structure among Rookeries of the Northwest Pacific Region

Most of the populations in this study revealed very low (F_{ST} = <0.05) genetic structuring through pairwise comparisons between rookeries – the majority of which were represented by statistically significant values of F_{ST} (Table 7). This indicates a very low level of population structuring (Wright et al., 1978). Recently, Roden et al. (2013) studied the genetic structure of regional green turtle stocks across the Pacific basin. The authors utilized 8 polymorphic microsatellite loci (including the six used in this study) and 20 single nucleotide polymorphisms (SNPs), which were used to analyze differentiation and gene flow among five rookeries throughout the Pacific basin. They reported moderate levels of differentiation (F_{ST} > 0.1) for the majority of comparisons throughout the Pacific, implying low gene flow among regions. One suggestion from this study was to include additional key Pacific rookeries to provide greater understanding of connectivity between nesting and foraging aggregations, in order to define stock boundaries. The low level of inter-rookery differentiation seen in this study suggests that breeding interactions are occurring among rookeries of this region. The results of this study have shown that the majority of pairwise comparisons of genetic differentiation within a regional scale are lower than reported values than between regions. This is concordant with a global study of green turtles that were genotyped at 4 polymorphic microsatellite loci by Roberts et al. (2004), which also provided evidence of higher inter- vs. intraoceanic population subdivision. A similar pattern was seen in Karl et al. (1992) in a global study of green turtle population structure using restriction fragment length polymorphisms (RFLPs).

Genetic differentiation between Lanyu and Wangan is very low (F_{ST} = 0.03),

contradicting a previous study by Cheng et al. (2008) using mtDNA, which discovered very high differentiation (FS_T = 0.673) between the island rookeries of Lanyu and Wangan, which lie on opposite sides of the main island of Taiwan. In that study, there were zero shared mtDNA haplotypes between the two rookeries, suggesting that these rookeries are genetically distinct, and that no gene flow occurs between them. This appeared to support their behavioural characteristics, as the results of tagging studies had not shown evidence of any movement of nesting females between the two islands. However, the results of this study indicate that these two populations have very low differentiation (F_{ST}=0.04181), with an estimated gene flow (*Nm*) of 5.73 migrants/generation. Discovering this previously undetected gene flow is largely due to the biparental inheritance of nuclear markers, and, contradictory to the findings of Cheng et al. (2008), indicates that breeding interactions occur between these two rookeries.

Following a similar pattern, a study by Nishizawa et al. (2011) discovered very high levels of differentiation between Lanyu and Iriomote (F_{ST}=0.4901) as well as with Ishigaki (F_{ST}=0.7529) using mtDNA. A similar pattern was seen between Wangan and Iriomote (F_{ST} = 0.1681) as well as with Ishigaki (F_{ST}=0.4381) in the same study. Post-nesting migrations of satellite-tracked female turtles from Wangan and Lanyu have provided evidence of the Ryukyu Archipelago of Southwestern Japan (including Iriomote and Ishigaki) being an important foraging and migratory area for turtles of these two Taiwanese rookeries (Cheng et al 2000, Wang et al, 2014). Studies in Japan have also identified the Ryukyu Archipelago as a nesting and foraging area for green turtles (Nishizawa 2013). This information can imply that turtles from rookeries of this area likely form a mixed-foraging aggregation. Due to the opportunistic mating behaviour of male turtles, it is likely that male-mediated gene flow occurs along foraging aggregations as well as migration routes, as has been predicted in other areas (Karl 1992). Evidence from this study appears to support the claim of gene flow occurs, as differentiation between Lanyu and Iriomote is very low (F_{ST}=0.0339), with a similar value for Ishigaki (F_{ST}=0.03968) with migration rates of 7.12 and 6.05 migrants per generation, respectively. A very similar pattern of very low differentiation was seen between Wangan and Iriomote (F_{ST}=0.03946) as well as Ishigaki (F_{ST}=0.03937), with gene flow (*Nm*) rates of 6.09 and

6.27, respectively. These similarities in gene flow values for Wangan and Lanyu and the Ryukyu islands are quite surprising, considering that a greater proportion of satellite-tagged females frequent the waters around Ryukyu, whereas satellite-tagged females from Wangan typically either disperse further northeast, toward the Kyushu archipelago, or southwest through the East China Sea (Cheng et al 2000, Wang et al, 2014).

Moderate differentiation occurs between Xiaoliuqiu and Iriomote (F_{ST}=0.06493) as well as Ishigaki (F_{ST}=0.05164), with gene flow (*Nm*) rates of 3.60 and 4.59 migrants/generation, respectively. This suggests that less gene flow occurs between Xiaoliuqiu compared to the other Taiwanese rookeries. A historically low nesting population size may play a factor in limited number of individuals for dispersal; however this is purely speculation, as historical data regarding nesting at this rookery simply does not exist.

The rookeries of Iriomote and Ryukyu exhibited very little genetic differentiation in a pairwise comparison of population differentiation (F_{ST} = 0.00634), with a very high rate of gene flow (*Nm*=39.17 migrants/generation). However, the F_{ST} value is not statistically significant from zero (Table 7), so these results should be interpreted with caution. Though the value itself is an order of magnitude lower in population structure, when compared to comparisons both within and represents the highest level of gene flow in this study.

Population Assignment Tests

Evidence for high levels of gene flow across all populations was also derived from Bayesian clustering analyses using the software STRUCTURE (Figure 5). Analysis of log-likelihood probability of the data revealed that the true number of genetic populations is K=1, which is supported by significantly low F_{ST} and *Nm* values across all populations.

The compiled results from CLUMPP and DISTRUCT output also supports this pattern of high admixture, as no distinct vertical clustering (indicating population structure) is seen

between populations at K=2, and individual probabilities of membership to any theoretical genetic population appears to be relatively even across all populations sampled (Figure 6). The same pattern is seen when the value of K is increased, with individuals exhibiting approximately equal probabilities of belonging to any given number of populations.

This finding is supported by the analysis of population differentiation and gene flow, as mating events occurring between rookeries results not only in low values of genetic divergence, but also the low individual assignment probabilities seen in the results from analysis using STRUCTURE.

Potential for Male-Mediated Gene Flow

A moderate level of male-mediated gene flow is suspected to occur when the breeding populations of genetically distinct rookeries overlap at feeding grounds or during migrations, regardless of whether males migrated to natal regions for breeding or not (Karl et al., 1992). Studies have suggested that highly reduced population structuring (low genetic differentiation) at nuclear markers relative to mitochondrial markers can be explained by male-mediated gene flow, i.e. Individuals mating between rookeries that are distinct according to mtDNA haplotype frequencies (Karl et al., 1992, FitzSimmons et al., 1997; Roberts et al., 2004; Roden et al., 2013).

Normally, levels of differentiation at nDNA and mtDNA should be compared directly, but are currently not yet available for this dataset. However, data from this study, and available F_{ST} values from mtDNA from Cheng et al. (2008) and Nishizawa et al. (2011) were compiled to provide a rough assessment of potential for male-mediated gene flow (Table 8). The comparison indicated that F_{ST} values among all comparisons were higher among mtDNA, by at least an order of magnitude at each pairwise comparison. While this is not an exact test by any means, this trend warrants further investigation by sequencing samples in this dataset at the mtDNA control region.

Data Considerations

Sample Size

When utilizing microsatellite loci, it is important to gain an accurate estimate of allele frequencies and diversity in order to determine population structuring. While large sample sizes are certainly ideal for any researcher, when studying endangered species it is often difficult to obtain large sample sizes due to the inherent difficulty in locating individuals as a result of low population sizes or densities. Hale et al. (2012) noted that detecting all of the alleles present within a population is less useful than obtaining an accurate estimate of allele frequencies and diversity, since very rare alleles are not particularly informative when assessing genetic diversity or population structure. So the goal should not necessarily be to determine all the alleles in a population, but rather the most representative set. The answer to what exact minimum sample size necessary for analysis of microsatellite data sets varies, however Hale et al.(2012) used a similar number of loci (between 5-9) to model optimal sample size (accuracy vs. cost), providing evidence that there is little benefit to sampling more than 25-30 individuals per population in studies such as this one. Of the five sampled populations, only three conformed to this minimum sample size: Lanyu (n=43), Iriomote (n=26) and Ishigaki (n=49). The explanation for a reduced sample size for Xiaoliuqiu is simple; there have not been enough nesting females present to sample in the years since sample collection began (2011). The explanation for reduced sample size in Wangan is the result of a partner on this project failing to provide results from remaining samples for incorporation into our analyses.

Conclusions and Recommendations

Results of analysis for population structure at 6 microsatellite loci polymorphic for green sea turtles have revealed extremely low population structure across 5 rookeries of the northwest Pacific region. The results strongly contrast past findings which implied that Wangan and Lanyu are distinct genetic populations, and exhibit no gene flow, while

providing evidence to support the existence of gene flow among all 5 rookeries. Population assignment tests using Bayesian clustering supported these findings by assigning all 5 rookeries analyzed in this study to a single genetic population. Assessment of the potential for male-mediated gene flow by contrasting mtDNA data from past studies, and nDNA F_{ST} values from this study suggests that male-mediated gene flow could possibly be occurring among these populations. This result is expected, considering the migratory patterns of nesting turtles at Wangan and Lanyu, the general patterns of habitat use by green turtles of this region, as well as the opportunistic mating behaviour of male green turtles seen in other populations worldwide.

Future recommendations include the incorporation of data from 57 samples taken from Wangan which had not yet arrive from a project partner. Furthermore, direct comparison of mtDNA control region at all of the samples could provide a robust test of the existence of male-mediated gene flow among rookeries. Also, satellite tracking of male turtles at all rookeries in this study could provide an element of ground-truthing for the results of these analyses, as currently, we know very little about the movements and interactions of male green turtles the northwest Pacific region.

These are green turtle populations of high conservation value, and representing nesting populations at the northern limit of their species range in the western Pacific. With the implication that the rookeries in this study represent a single breeding population, sea turtle stock management should incorporate a multi-national approach that represents the international movements and interactions of these endangered reptiles, in order to ensure the future of this critical population.

References

Balazs, G.H. 1982. Growth rates of immature green turtles in the Hawaiian archipelago. K.A. Bjorndal [ed.]. Biology and Conservation of Sea Turtles. Smithsonian Institution Press. Washington, pp. 118-125.

Balazs, G. H., and M Chaloupka. 2004. Thirty-year recovery trend in the once depleted Hawaiian green sea turtle stock. Biological Conservation 117: 491-498.

Balloux, F., and N. Lugon-Moulin. 2002. The estimation of population differentiation with microsatellite markers. Molecular Ecology 11: 155-165.

Bass, A. L., S.P. Epperly, and J. Braun-McNeill. 2006. Green turtle (*Chelonia mydas*) foraging and nesting aggregations in the Caribbean and Atlantic: impact of currents and behavior on dispersal. Journal of Heredity 97: 346-354.

Botstein, D., R.L. White, M. Skolnick, and R.W Davis. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. American Journal of Human Genetics 32, 314–331.

Bowen, B. W., F.A. Abreu-Grobois, G.H. Balazs, N. Kamezaki, C.J. Limpus, and R.J. Ferl 1995. Trans-Pacific migrations of the loggerhead turtle (*Caretta caretta*) demonstrated with mitochondrial DNA markers. Proceedings of the National Academy of Sciences: 92: 3731-3734.

Bowen, B.W. and S.A. Karl . 2007. Population genetics and phylogeography of sea turtles. Molecular Ecology 16: 886-4907.

Carr, A. and A.B Meylan. 1980. Evidence of passive migration of green turtle hatchlings in *Sargassum*. Copeia 1980: 366-368.

Chapuis M.P., A. Estoup. 2007. Microsatellite Null Alleles and Estimation of Population Differentiation. Molecular Biology and Evolution 24: 621-631.

Cheng, I. J. 1996. Sea turtles at Taipin Tao, South China Sea. Marine Turtle Newsletter, 75, 6-8.

Cheng, I. J. 2000. Post-nesting migrations of green turtles (*Chelonia mydas*) at Wan-an Island, Penghu Archipelago, Taiwan. Marine Biology 137: 747-754.

Cheng, I. J., Dutton, P. H., Chen, C. L., Chen, H. C., Chen, Y. H., and J.W. Shea. 2008. Comparison of the genetics and nesting ecology of two green turtle rookeries. Journal of Zoology 276: 375-384.

Cheng, I. J., C.T. Huang, P.Y. Hung, B. Z. Ke, C.W. Kuo, and C. L. Fong. 2009. Ten years of monitoring the nesting ecology of the green turtle, *Chelonia mydas*, on Lanyu (Orchid Island), Taiwan Zoological Studies 48: 83-94.

Cole, C.T. 2005. Allelic and population variation of microsatellite loci in aspen (*Populus tremuloides*). New Phytologist 167: 155-164.

Crouse, D.T., L. B. Crowder, and H. Caswell. 1987. A stage-based population model for loggerhead sea turtles and implications for conservation. Ecology 68:1412 – 1423.

Dakin, E.E., Avise, J.C. 2004. Microsatellite null alleles in parentage analysis. Heredity 93: 504–509.

Day, I.N.M. 2001. “Molecular Genetic Epidemiology: A Laboratory Perspective” Cambridge, Springer-Verlag, Berlin, Heidelberg, New York.

Dutton P.H., and A. Frey. 2009. Characterization of polymorphic microsatellite markers for the green turtle (*Chelonia mydas*). Molecular Ecology Resources 9: 354−356.

Earl, D.A. and B.M. von Holdt. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4: 359-361.

Ellis, J. S., J. Gilbey, A. Armstrong, A., Balstad, T., Cauwelier, E., Cherbonnel, C., J. R. Stevens. 2011. Microsatellite standardization and evaluation of genotyping error in a large multi-partner research programme for conservation of Atlantic salmon (*Salmo salar* L.).Genetica 139(3), 353–367.

Ergine, S., C. Aymak, and A.H. Ucar. 2011. Carapacial scute variation in green turtle (*Chelonia mydas*) and loggerhead turtle (*Caretta caretta*) hatchlings in Alata, Mersin, Turkey. Turkish Journal of Zoology 35: 343-356.

Excoffier, L., and H.E.L. Lischer. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources. 10: 564-567.

Falush, D., M. Stephens, and J.K. Pritchard. 2003. Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. Genetics 164: 1567–1587.

FitzSimmons, N. N., Moritz, C.J Limpus, L. Pope, and R. Prince. 1997. Geographic structure of mitochondrial and nuclear gene polymorphisms in Australian green turtle populations and male-biased gene flow. Genetics 147: 1843-1854.

Gill, P., C. Kimpton, and E. D'aloja. 1994. Report of the European DNA profiling group (EDNAP) - towards standardization of short tandem repeat (STR) loci. Forensic Science International 65: 51–59.

Glaubitz, J.C. 2004. Convert: A user-friendly program to reformat diploid genotypic data for commonly used population genetic software packages. Molecular Ecology Notes 4: 309-310.

Guo, S. W., and E.A. Thompson. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics, 361-372.

Hale, M.H., T.M. Burg, T.E. Steeves. 2012. Sampling for Microsatellite-Based Population Genetic Studies: 25 to 30 Individuals per population Is Enough to Accurately Estimate Allele Frequencies. PloS ONE 7: e45170.

Hashiyada, M. 2011. DNA biometrics,Biometrics. Dr. Jucheng Yang (Ed.), ISBN: 978-953-307-618-8, InTech.

Jakobsson, M. and N.A. Rosenberg. 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23: 1801-1806.

Jensen, M.P., N. FitzSimmons, and P.H. Dutton. 2013. Molecular Genetics of Sea Turtles. In P.L. Lutz and J.A. Musick (eds) The Biology of Sea Turtles: Volume III, pp. 135-162. CRC Press, Boca Raton, Florida.

Kalinowski, S.T., M.L. Taper, and T.C. Marshall. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular Ecology 16: 1099-1106.

Karl, S. A., B.W. Bowen, and J. C. Avise. 1992. Global population genetic structure and male-mediated gene flow in the green turtle (*Chelonia mydas*): RFLP analyses of anonymous nuclear loci. Genetics 131: 163-173.

Lewison, R. L., and L. B. Crowder. 2007. Putting longline bycatch of sea turtles into perspective. Conservation Biology 21: 79-86.

Limpus, C.J. 2009. A Biological Review of Australian Marine Turtles: 2 Green Turtle (Chelonia mydas) (Linneaus). State of Queensland, Australia: Environmental Protection Agency. ISBN 978-0-9803613-2-2.

Luschi P, G.C. Hays, F. Papi. 2003. A review of long-distance movements by marine turtles, and the possible role of ocean currents. Oikos 103:293–302.

Meylan, A.B., B.W. Bowen, and J.C. Avise. 1990. A genetic test of the natal homing versus social facilitation models for green turtle migration. Science 248: 724-727.

Musick, J.A., and C.J. Limpus. 1997. Habitat utilization and migration in juvenile sea turtles. In: P.L. Lutz and J.A. Musick (eds) The Biology of Sea Turtles, pp. 137-164. CRC Press, Boca Raton, Florida.

Naro-Maciel, E., Le, M., FitzSimmons, N. N., and G. Amato. 2008. Evolutionary relationships of marine turtles: a molecular phylogeny based on nuclear and mitochondrial genes. Molecular Phylogenetics and Evolution, 49: 659-662.

Pritchard, J.K., M. Stephens, P.J. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Pritchard, P. C. H. and J.A. Mortimer. 1999. Taxonomy, External Morphology, and Species Identification. pp. 21-38. In: Eckert, K.L., K.A. Bjorndal, F.A. Abreu-Grobois, and M. Donnelly [eds.]. Research and Management Techniques for the Conservation of Sea Turtles. IUCN/SSC Marine Turtle Specialist Group Publication No. 4.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity 86 : 248-249.

Roberts, M. A., T.S. Schwartz, and S.A. Karl. 2004. Global population genetic structure and male-mediated gene flow in the green sea turtle (Chelonia mydas): analysis of microsatellite loci. Genetics: 166: 1857-1870.

Roden, S. E., P. A. Morin, A. Frey, G. H. Balazs, P. Zarate, I.-J. Cheng, and P. H. Dutton. 2013. Green turtle population structure in the Pacific: New insights from SNPs and microsatellites. Endangered Species Research 20 :227–234.

Selkoe, K. A. and R.J. Toonen. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecology Letters 9: 615–629.

Seminoff, J.A. 2004. *Chelonia mydas*. The IUCN Red List of Threatened Species. Version 2014.3. <www.iucnredlist.org>. Downloaded on 07 July 2014.

Shinde D., Y.L. Lai, F.Z. Sun, and N. Arnheim. 2003. Taq DNA polymerase slippage mutation rates measured by PCR and quasi-likelihood analysis: (CA/GT)(n) and (A/T)(n) microsatellites. Nucleic Acid Research 31: 974–980.

Smit-McBride, Z., A. Moya, and F.J. Ayala. 1988. Linkage disequilibrium in natural and experimental populations of *Drosophila melanogaster*. Genetics 120: 1043-1051.

Stewart, S., D.Wickramasinghe, A.E. Dorrance, and E.R. Alison. 2011. Comparison of three microsatellite analysis methods for detecting genetic diversity in *Phytophthora sojae* (Stramenopila: Oomycete). Biotechnology letters 33: 2217-2223.

Troëng, S., and E. Rankin 2005. Long-term conservation efforts contribute to positive green turtle *Chelonia mydas* nesting trend at Tortuguero, Costa Rica. Biological Conservation,121(1), 111-116.

van Houtan, K.S., S.K. Hargrove, and G.H. Balazs. 2014. Modeling Sea Turtle Maturity from Partial Life History Records. Pacific Science 68: 465-477.

van Oosterhout C.V., W.F. Hutchinson, and D.P.M., Shipley P (2004) MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes. 4: 535-538.

van Oosterhout C., D. Weetman, and W.F. Hutchinson. 2006. Estimation and adjustment of microsatellite null alleles in nonequilibrium populations. Molecular Ecology Notes 6: 255–256.

Wang, H. C., & I. J. Cheng. 1999. Breeding biology of the green turtle, *Chelonia mydas* (Reptilia: Cheloniidae), on Wan-An Island, PengHu archipelago. II. Nest site selection.Marine Biology 133: 603-609.

Whittow, G. C., and G. H. Balazs.1982. Basking behavior of the Hawaiian green turtle (*Chelonia mydas*). Pacific Science 36: 129-139.

Wattier R., C.R. Engel, P. Saumitou-Laprade, and M. Valero 1998. Short allele dominance as a source of heterozygote deficiency at microsatellite loci: experimental evidence at the dinucleotide locus Gv1CT in *Gracilaria gracilis* (Rhodophyta). Molecular Ecology 7: 1569–1573.

Weir, B.S., Cockerham, C.C. 1984. Estimating F-statistics for the analysis of population-structure. Evolution 38: 1358−1370.

Whitlock, M. C., and D.E. McCauley. 1999. Indirect measures of gene flow and migration: FST≠ 1/(4*Nm*+ 1). Heredity: 82: 117-125.

Wright, S. 1978. Evolution and the genetics of population, variability within and among natural populations. Univ. of Chicago Press, Chicago.

Figures

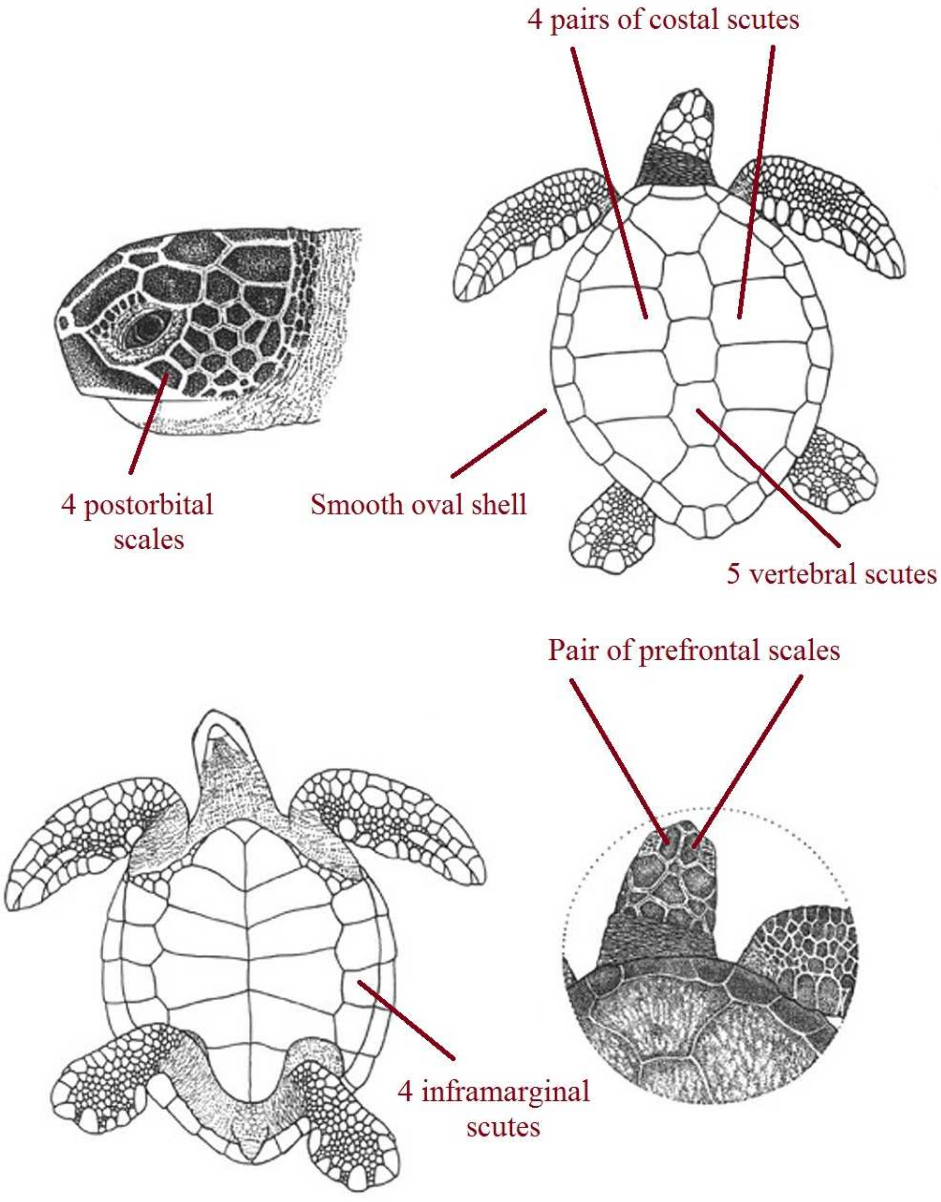

Figure 1 – Green turtle external morphology (modified from Pritchard and Mortimer 1999).

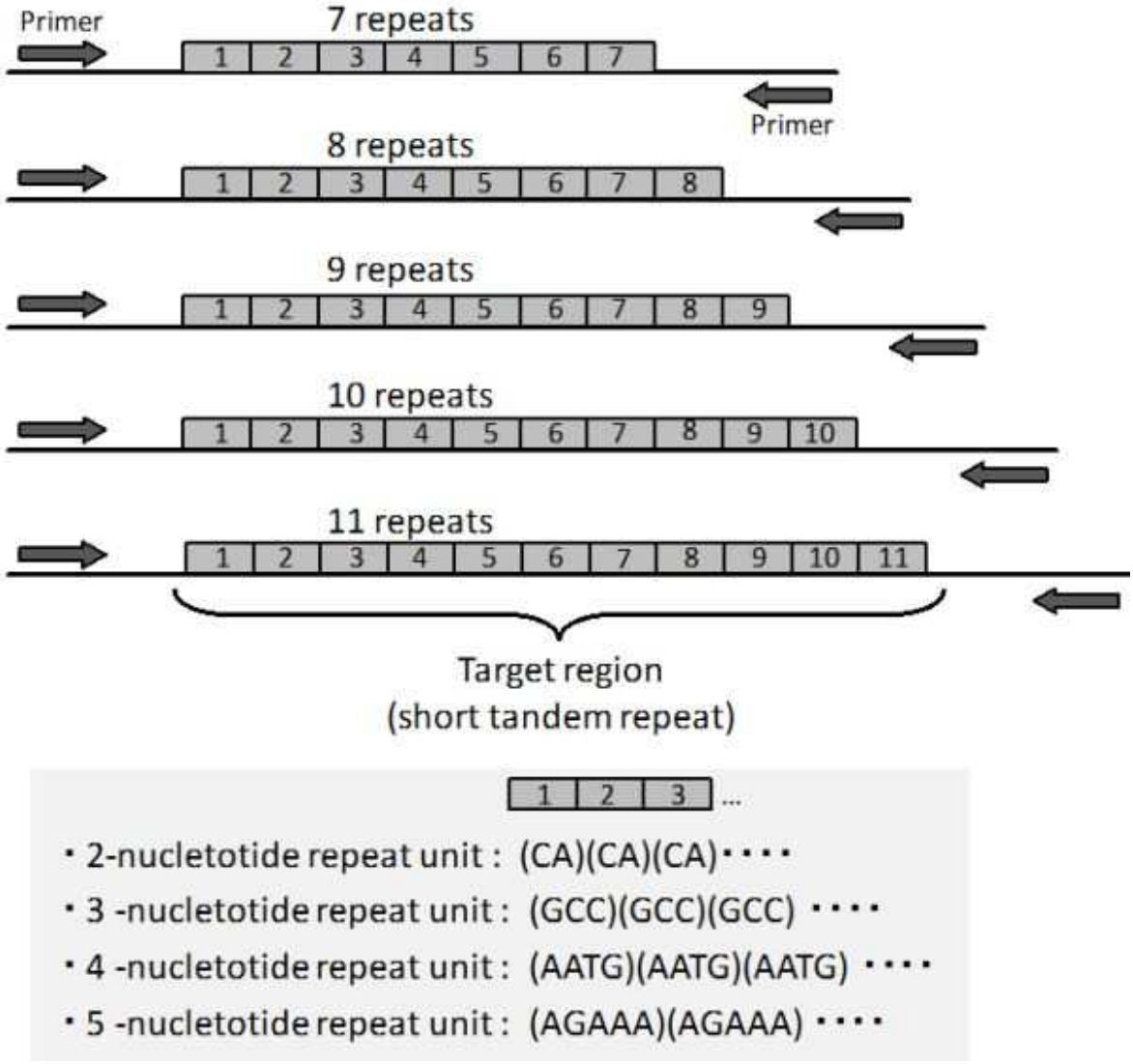

Figure 2 - Simplified diagram of a microsatellite (STR) region, primer locations and repeat characteristics (from Hashiyada 2011).

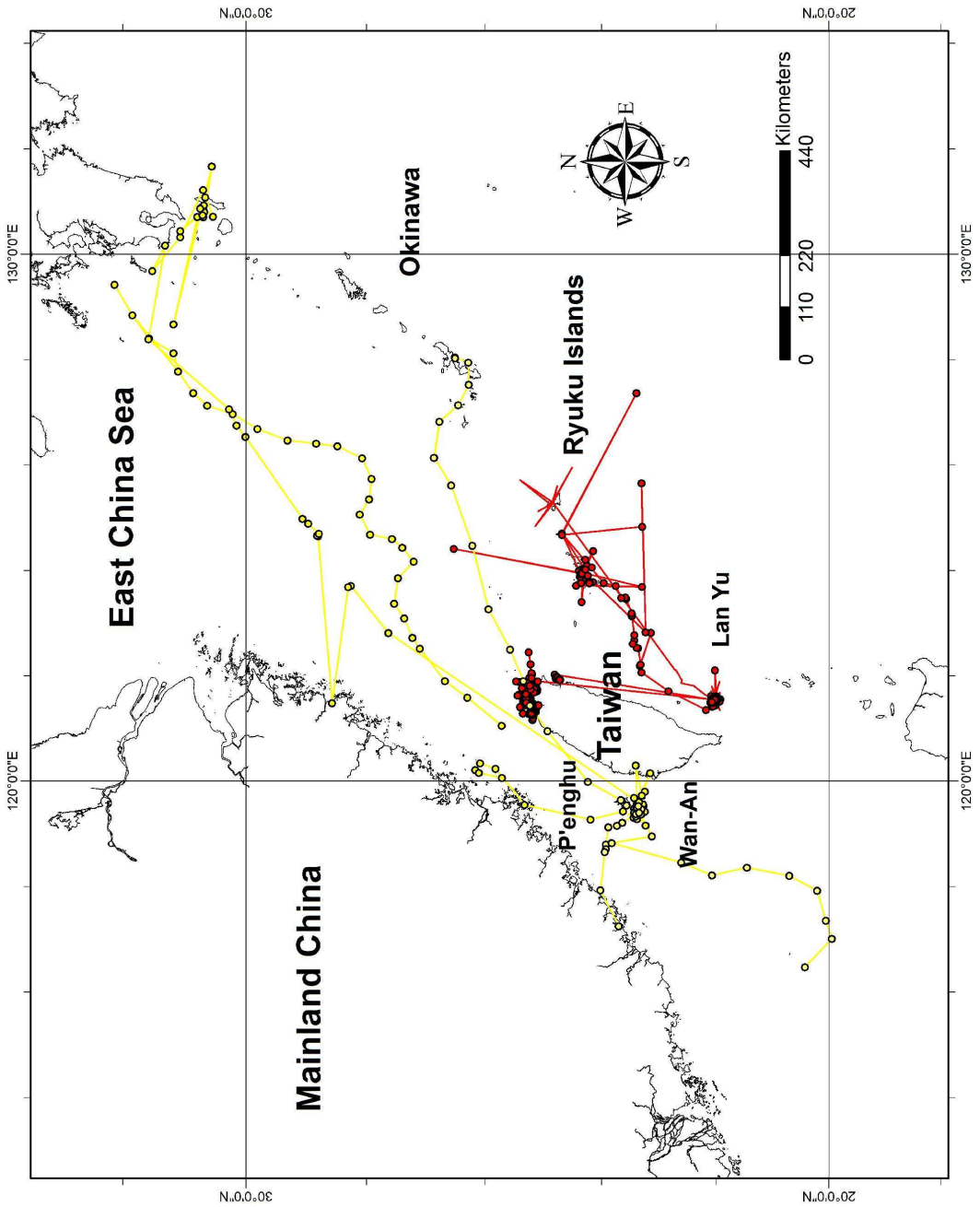

Figure 3 – Movements of satellite-tagged green turtles tracked from Wangan and Lanyu (map created using data from Cheng et al. 2000 and Wang et al. 2014). Map provided by W-H Cheng.

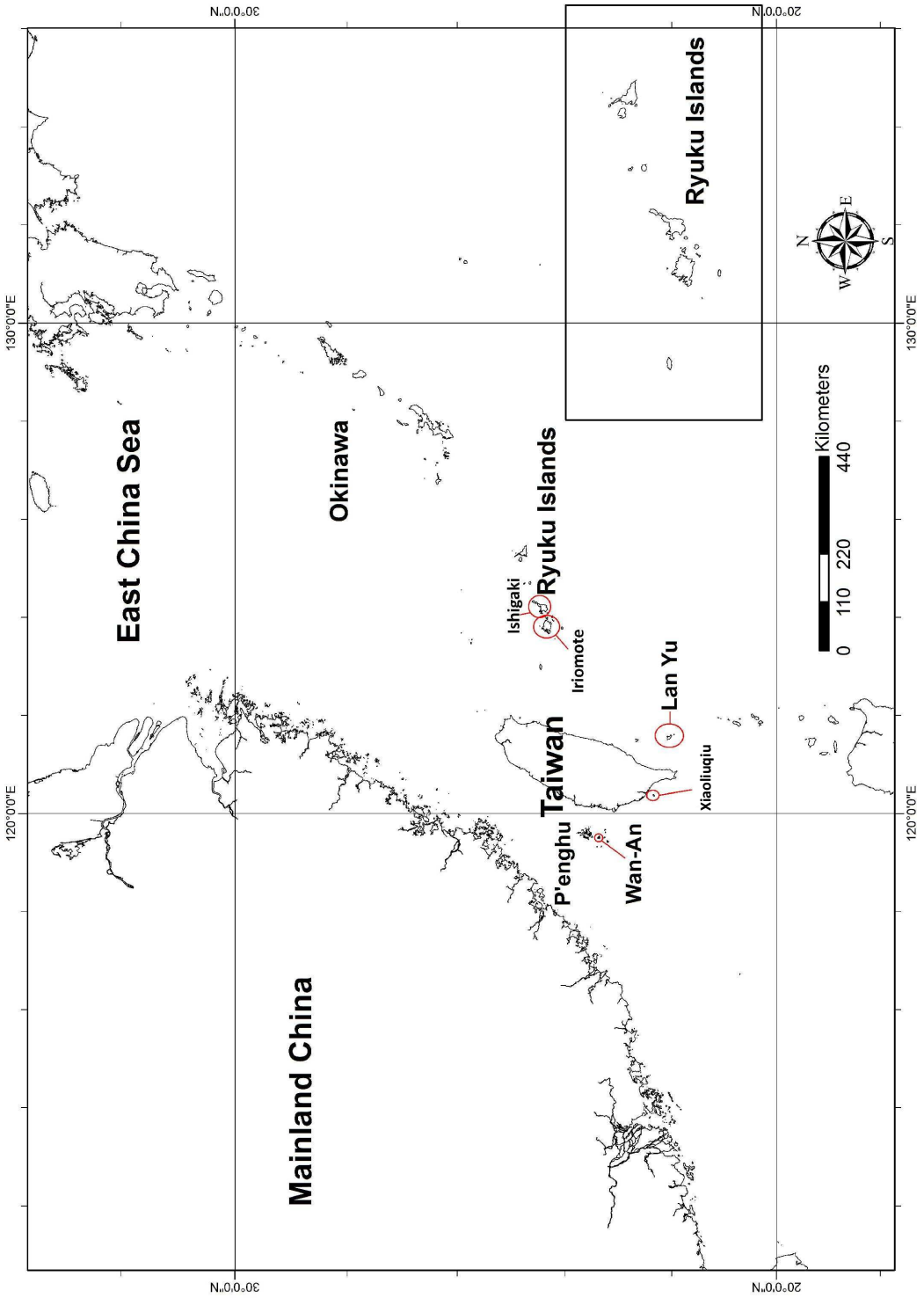

Figure 4 – *C. mydas* rookeries of the northwest Pacific sampled in this study. Map provided by W-H Cheng.

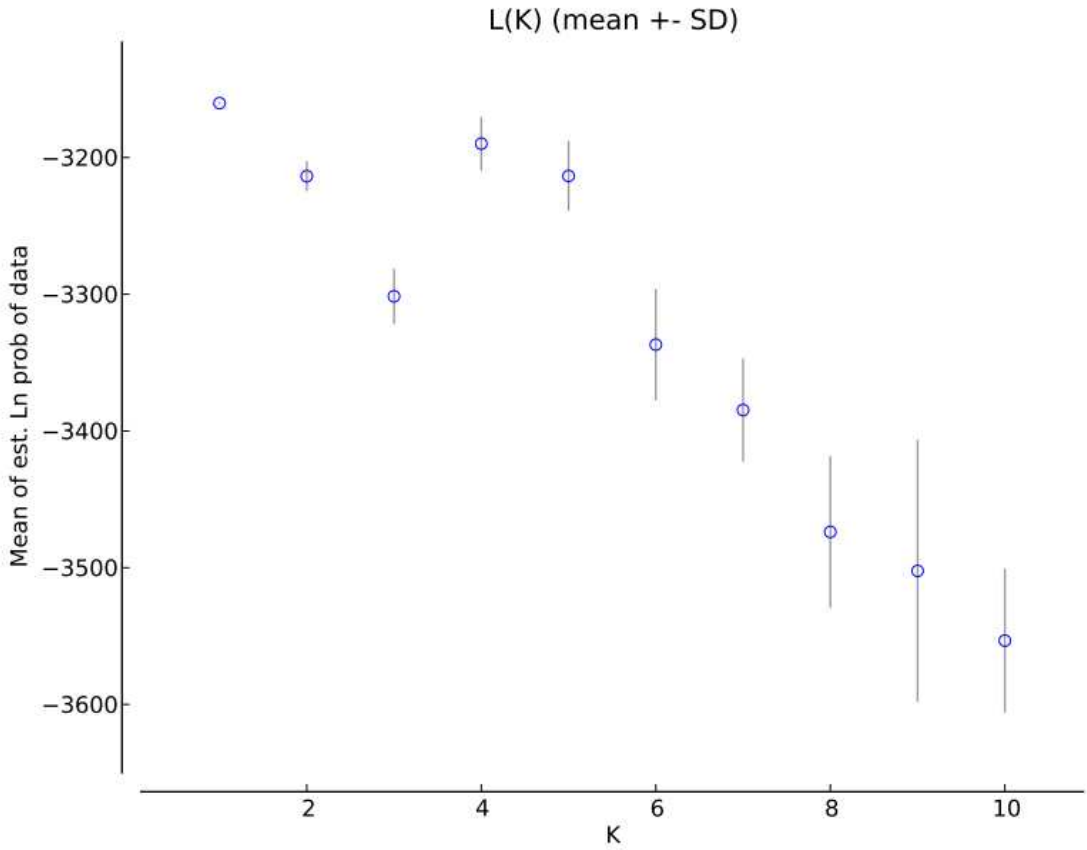

Figure 5– Mean log-likelihood values (±SD) for five replicate runs of 500,000 iterations for each value of K (max # of populations) from STRUCTURE software.

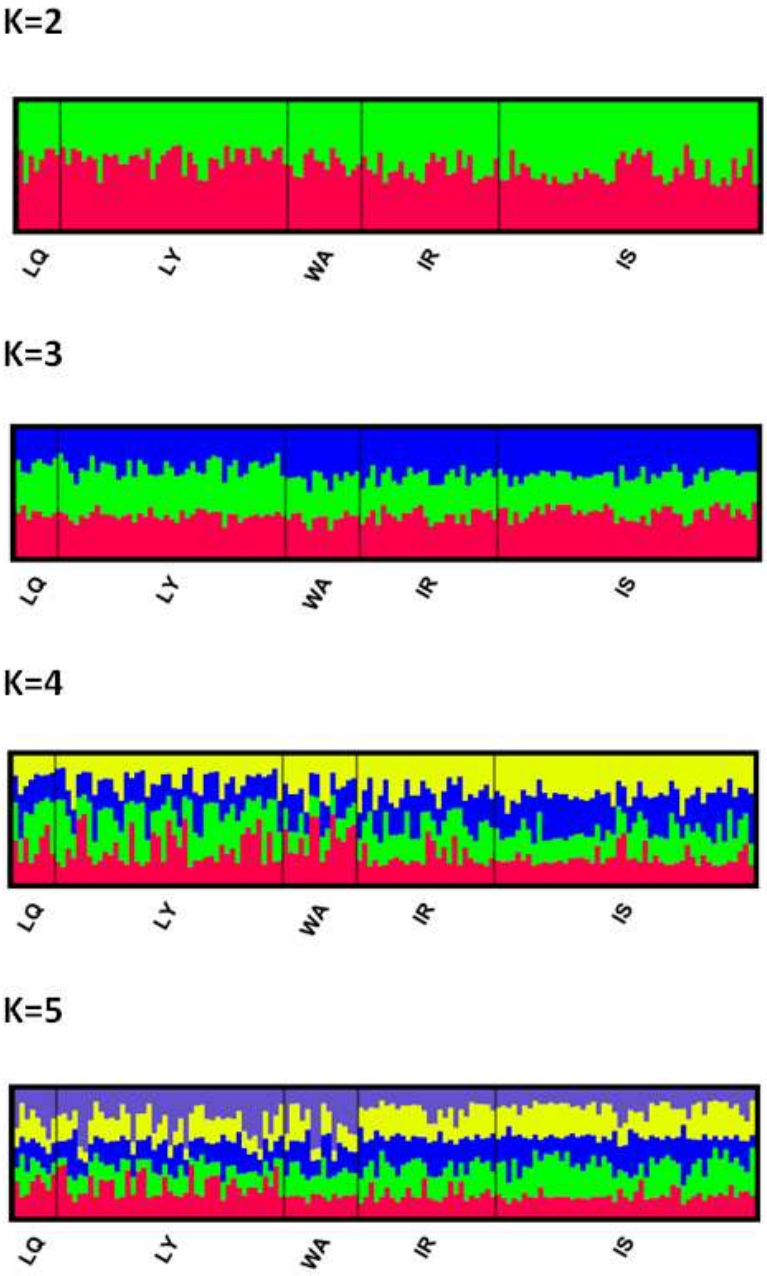

Figure 6– Bar plots from STRUCTURE. Data from structure output was assigned a color code for each genetic cluster (i.e. theoretical population). Each labelled column represents a sampled rookery, and each sub-column represents individuals and their probability of belonging to a theoretical population (colour). Labels are: LQ= Xiaoliuqiu, LY = LanYu, WA= WangAn, IR= Iriomote and IS= Ishigaki.

Tables

Table 1 – Summary of the six microsatellite loci from Dutton and Frey (2009) and Roden et al. (2013) that were utilized in this experiment.

Locus	Primer sequence	Repeat motif	Fluourescent Dye used (Taiwan/Japan)	Expected Size range (bp)	Genbank accession number
A6F	AGTGCAATAACCATCCTTACAC	GA_{16}	ROX / D4	115-135	EU668882
A6R	GGGCTGAATAGAGCTACAGAC				
B108F	GCCATTCTTCTTTCATTCCT	CAA_{7}	HEX / D4	181-193	EU668884
B108R	GTTTCTAGCATTACTCACCTGTCTTAGC				
D1F	CAGGCTGATTATGCTTTGTT	$TAGA_{14}$	ROX / D4	204-264	EU668888
D1R	GTTTCTAAGGGAAACTGATTCTCTGG				
D2F	AGTCCCCACTACTCATACCC	$TAGA_{10}$	HEX / D4	283-328	EU668889
D2Rt	GTTTCTTTTGTGTTACTTCGGTGTTTC				
D102F	TCATGGATCTTAAAAGCACAG	$TAGA_{8}$	FAM / D4	154-166	EU668898
D102R	TTTCTGGACATCAGGAGAGTAT				
D115F	AACTGCTACTGGTCATTCTTCA	$TAGA_{21}$	TAMRA / D4	147-213	EU668893
D115R	GTTTCTGCTGCCTCTTCTAAAAGGAG				

Table 2 – Number of samples collected from nesting female *C. mydas* in Taiwan (TW) and southwestern Japan (JP), where n = sample size.

Sampling Site	n
Xiaoliuqiu (TW)	8
Wangan (TW)	14
Lanyu (TW)	43
Iriomote (JP)	26
Ishigaki (JP)	49

Table 3 – Amplification success at 6 polymorphic microsatellite loci for 140 *C. mydas* samples.

Locus	Amplification success (%)
A6	99.3
B108	97.1
D1	96.4
D1	96.4
D102	98.6
D115	91.4

Table 4 – Summary statistics at 6 microsatellite loci based on 140 *C. mydas* in the northwest Pacific region where n= the number of successfully amplified samples per locus, k= number of alleles per locus, Ho = observed heterozygosity, He = expected heterozygosity, PIC = polymorphic information content, and HWE = conformity to Hardy-Weinberg Equilibrium.

Locus	n	k	Ho	He	PIC	HWE
A6	139	10	0.914	0.862	0.844	Yes
B108	136	7	0.478	0.788	0.756	No
D1	135	14	0.666	0.858	0.841	No
D2	135	16	0.815	0.876	0.860	No
D102	138	5	0.391	0.380	0.336	Yes
D115	128	21	0.867	0.917	0.907	Yes

Table 5 – Summary statistics for 5 populations of *C. mydas* in the northwest Pacific analyzed at 6 microsatellite loci, where n= sample size, Ho= observed heterozygosity, He= expected heterozygosity, k= mean allelic richness and HWE = conformity to Hardy-Weinberg Equilibrium. Standard error (±) is in brackets.

Population	n	Ho	He	k	HWE
Xiaoliuqiu	8	0.577 (0.299)	0.742 (0.120)	4.50 (1.64)	Yes
Lanyu	14	0.597 (0.295)	0.742 (0.244)	9.00 (3.85)	No
Wangan	43	0.600 (0.385)	0.756 (0.197)	6.50 (2.74)	No
Iriomote	26	0.779 (0.224)	0.772 (0.207)	8.83 (3.25)	No
Ishigaki	49	0.765 (0.138)	0.742 (0.173)	10.00 (4.60)	No

Table 6 - Results of tests for linkage disequilibrium from Arlequin v3.5.1.3. An "x" denotes significant ($p<0.05$) linkage between loci.

Locus	A6	B108	D1	D2	D102	D115
A6	-					
B108	x	-				
D1	x	x	-			
D2	x	x	x	-		
D102	x	x		x	-	
D115	x			x	x	-

Table 7 – Genetic differentiation (F_{ST}-in bold, above) and number of migrants per generation (*Nm* – in italics, below) of *C. mydas* among five sampled nesting populations at 6 microsatellite loci.

	Xiaoliuqiu	Lanyu	Wangan	Iriomote
Xiaoliuqiu				
Lanyu	0.02407 10.14			
Wangan	0.05828* 4.04	0.04181* 5.73		
Iriomote	0.06493* 3.60	0.03391* 7.12	0.03946* 6.09	
Ishigaki	0.05165* 4.59	0.03968* 6.05	0.03837* 6.27	0.00634 39.17

* p = <0.05

Table 8 – Comparison of FST values for 6 microsatellite loci and mtDNA control region at 4 *C. mydas* rookeries in the northwest Pacific region to assess potential for male-mediated gene flow. Format: (microsatellite FST / mtDNA FST) Values in **bold** indicate significant FST values.

	Lanyu	**Wangan**	**Iriomote**
Lanyu			
Wangan	**0.0418 / 0.673** [(a)]		
Iriomote	**0.0339** / **0.4901** [(a)(b)]	**0.0395 / 0.1681** [(a)(b)]	
Ishigaki	**0.0397 / 0.7529** [(a)(b)]	**0.0384 / 0.4381** [(a)(b)]	0.00634 / **0.1578** [(b)]

[(a)] – Source: (Cheng et al., 2008)
[(b)] – Source: (Nishizawa et al., 2011)