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以粒線體 DNA 及雙限制酶切位點標定法探究臺灣小燕鷗的遺傳多樣性及族群結構

Exploring genetic diversity and population structure of the Little Tern (*Sternula albifrons*) in Taiwan based on mtDNA and ddRAD sequencing data

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本論文係 江美雪（R09241215）在國立臺灣大學海洋研究所完成之碩士學位論文，於民國 111 年 12 月 30 日承下列考試委員審查通過及口試及格，特此證明。

The undersigned, appointed by the Institute of Oceanography on 30th December 2022 have examined a Master's thesis entitled above presented by Mei-Shuet Kong (R09241215) candidate and hereby certify that it is worthy of acceptance.

口試委員 Oral examination committee:

（指導教授 Advisor）

所長 Director:

致謝

首先，我想感謝我的指導教授陳韋仁老師這兩年半的指導。在老師耐心地指下，讓我能更深入了解族群遺傳學的基本理論及懂得如何以族群遺傳的角度解釋分子資料，使我受益匪淺。老師也總是在我有困難時，及時向我伸出援手，包括幫助我尋找能獲得獎助學金的管道，減少我在經濟上的壓力，讓我在學習上能更無後顧之憂。當我提出在外縣市採樣上遇到的交通問題時，老師更費盡心力地尋找解決方法，甚至跟我一起出差到澎湖外島採樣。此外，我非常感謝實驗室的助理們包括秀貞姐、思璿學長、德婇及學長姐包括李欣學姐、洪君學長、凌藍學姐及文君學姐，他們常常特意騰出時間教導及帶領我實驗上的操作，讓我能更容易理解實驗的流程及更順利地完成我的實驗。在學長姐的用心指導下，我也學習及接觸了很多以前從沒接觸過的軟件以更有效的分析我的研究數據。

我也萬分感謝森林系的袁孝維老師以及其團隊（洪崇航及陳韋廷學長等）提供資源及研究上的指導，尤其是在採樣上的協助，他們總是毫無怨言、不遺餘力地陪伴並幫助我到不同的採集地採樣，並時刻關注我的採樣數量是否足夠和過程是否順利。同時，我也感謝澎湖、宜蘭及花蓮鳥會於採樣上給我的建議和協助，讓我在採樣時能更順暢無阻。在此也感謝許育誠老師、林思民老師及丁宗蘇老師撥冗審閱論文，給予寶貴意見，使得論文更加完善。最後，我想感謝我的家人朋友們在我進行研究及採樣和撰寫論文遇到挫折時，總是給我各種建議及無限的鼓勵，讓我總是能再次回憶起當初想來求學的初衷並激起鬥志，重新打起精神一步一步地順利完成我於研究中的目標！

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中文摘要

近幾十年來，由於棲息地喪失和人為的干擾，包括小燕鷗（*Sternula albifrons*）在內的海鳥族群數量一直在下降。小燕鷗目前被列為臺灣的二級保育類鳥種（也就是，珍貴稀有物種），是鷗科中唯一在台灣本島有繁殖地的物種。海鳥的族群遺傳多樣性及結構之研究有助於規劃保育策略及相關的物種的保護單位，然而，這種對海鳥，特別是台灣小燕鷗的族群遺傳之相關資訊仍缺乏。因此，本研究利用長度為 861-bp 的粒線體 DNA 控制區及使用雙限制酶切位點標定法（ddRAD）篩選出的 5113 個單核苷酸多態性（SNPs）為資料進行臺灣小燕鷗的族群遺傳研究。本研究於四個已知的小燕鷗繁殖地，分別爲臺灣西側的澎湖和彰化以及臺灣東側的宜蘭和花蓮，採集共 59 個幼鳥羽毛樣本。由於海鳥族群之分化可能受其不同族群遷徙路線的影響，而經臺灣遷徙的海鳥也可能沿西側與沿東側海岸通過。因此，本研究將樣本亦再依採集地分為兩個地理組（即臺灣西部和東部）以進一步探討臺灣小燕鷗於臺灣西側與東側間的族群是否有差異及評估其在區域上的差異程度。在進行族群遺傳相關的分析前，本研究也利用粒線體細胞色素 c 氧化酶 I（*COI*）基因序列確認野外採集樣本的物種及臺灣小燕鷗的亞種名稱定義。本研究意外發現其中一個從宜蘭採集的樣本的 *COI* 基因序列與美洲小燕鷗（*S. antillarum*）極度相似，並在後續分析中確認。美洲小燕鷗與小燕鷗有相近的親緣關係，但因美洲小燕鷗只分佈於北美和南美，故此推斷為該美洲小燕鷗的親鳥可能因遷徙時遭遇不好的氣候條件，迷失原遷徙路線而意外跟隨小燕鷗族群來到臺灣進行繁殖。本研究中利用粒線體 DNA 控制區序列進行的族群遺傳分析與 ddRAD 序列的分析結果相互支持對應。粒線體 DNA 控制區序列的單型網狀圖（haplotype network）及 ddRAD 序列的集群分析 (clustering analyses) 均無法根據採集地以區分臺灣西側及東側的樣本為兩群。此外，利用兩種序列進行的 AMOVA 分析和計算的各群間的遺傳分化指數（pairwise Φ_{ST}/F_{ST}）顯示臺灣西側與東側的小燕鷗族群處於近乎零的基因分化情形，顯示出西側與東側的小燕鷗族群間有著高度的連通性。另外，本研究也將臺灣小燕鷗的粒線體DNA控制區序列與日本小燕鷗的相同基因序列合併並加以分析以比較臺灣和日本小燕鷗的族群遺傳多樣性的差異及探討兩者之間的族群連通性。單型網狀圖無法區分臺灣和日本的樣本為不同的地理區群集，但圖中三個高頻率出現的共同單倍型（common

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haplotype）均各別多數出現來自臺灣、日本冲繩及日本本島的樣本。AMOVA 和各群間的遺傳分化指數也顯示臺灣、日本冲繩及日本本島的小燕鷗處於中至強度的族群分化情形。小燕鷗在臺灣及日本的族群間具有距離隔離（isolation by distance, IBD）現象，也就是族群間的遺傳距離會與地理距離成顯著正相關。本研究亦針對小燕鷗在臺灣及在日本的族群之族群遺傳分析結果進行比較及討論，並依據研究經驗及結果提出未來小燕鷗在臺灣可供參考的保育策略。

關鍵詞：小燕鷗、族群遺傳、粒線體DNA控制區、雙限制酶切位點標定法、單核苷酸多態性、保育、臺灣

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Abstract

In recent decades, seabird populations including the Little Tern (*Sternula albifrons*) populations have been declining due to habitat loss and human disturbance. The Little Tern is currently listed as the class II species (rare and valuable species) under the Wildlife Conservation Act Republic of China (Taiwan) and it is the only species in the family Laridae who has breeding records in mainland Taiwan. Examining the genetic diversity and population structure of seabirds can help on managing the conservation units of the particular species. However, such kind of the population genetic analyses on seabirds, particularly the Little Tern in Taiwan remains poorly conducted. In this study, the Little Tern populations in Taiwan were examined based on two different types of data: the mitochondrial (mt) control region (D-loop) DNA sequences (861 bp) and double digest Restriction-site Associated DNA (ddRAD) sequencing data with 5113 single nucleotide polymorphisms (SNPs) generated. The feather samples were collected from 59 chicks of the Little Tern across four known breeding colonies located at western (Penghu and Zhanghua) and eastern (Yilan and Hualien) coasts of Taiwan. Since seabird populations can be shaped by their migratory routes and two main migrations along western and eastern coastal lines in Taiwan might be suspected, the studied populations were further grouped into two geographical groups (i.e., West and East of Taiwan) to determine the degree of population differentiation at regional scale. Before conducting the population genetic analyses, the mitochondrial Cytochrome c oxidase I (*COI*) gene (or DNA barcoding gene) sequence information of the samples were extracted and analysed to confirm the field identification and the subspecies status of the Little Tern in Taiwan. The phylogenetic analysis using *COI* sequences revealed that one individual collected from Yilan was highly matched to the Least Tern (*Sternula antillarum*), a congeneric species of the Little Tern which only breeds in the North and South America.

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The parents of this individual were believed to get lost due to bad weather while migrating to their breeding grounds in America and accidentally followed the Little Tern colonies to breed in Taiwan. As to the population genetic analyses, the results obtained based on D-loop sequences were consistent with the results obtained based on ddRAD sequencing data. The mtDNA haplotypes constructed based on D-loop sequences and the clustering analyses conducted based on ddRAD sequencing data did not cluster the samples into two geographical groups with respect to the West and East of Taiwan. Furthermore, the AMOVA analyses and pairwise Φ_{ST} /F_{ST} estimations based on both types of data revealed little to no population differentiation among populations and between regions. The findings of this study suggested a high population connectivity among the breeding colonies in Taiwan. Additionally, the obtained D-loop sequences of the Little Tern from Taiwan were compiled with those from Japan deposited in the NCBI GenBank to compare the genetic diversity and to examine the phylogeographic break that may shape the diversity of the Little Tern populations in eastern Asia. The resulting haplotype network did not clearly separate Taiwanese and Japanese populations but the three most common haplotypes were prevalent for mainland Japan, Okinawa and Taiwan samples, respectively. The Little Tern populations may be frequently connected but with some restrictions on their gene flow that caused moderate to great differentiation among the three populations, which further supported by the AMOVA analyses, pairwise Φ_{ST} estimations and the positive yet significant isolation by distance (IBD) pattern. The possible explanations leading to little evidence for genetic structure of the Little Tern populations in Taiwan and moderate to strong structure among Taiwan, Okinawa and mainland Japan populations were discussed. The concerns for the Little Tern protection in Taiwan were also discussed to recommend the conservation strategies of this species in Taiwan.

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Keywords

Little Tern, population genetics, mtDNA control region, ddRAD, SNP, conservation, Taiwan

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Contents

doi:10.6342/NTU202300368

List of Figures

doi:10.6342/NTU202300368

List of Tables

doi:10.6342/NTU202300368

Chapter 1 Introduction

For over half a century, seabird populations have been severely decreasing, especially in wide-ranging species (Paleczny et al 2015). The Little Tern (*Sternula albifrons*, Pallas 1794) is an example of wide-ranging species which is distributed throughout the world's continents except for Antarctica and the Americas (Olsen and Larsson 1995). The populations of this species are generally in declining trend globally due mainly by habitat loss and degradation, predation and human disturbances (BirdLife International 2022). Declining populations of the Little Tern has made it received some conservation concerns in some regions. For instance, it is listed as an endangered species in New South Wales, Queensland, and Tasmania of Australia (BirdLife Australia 2022); it is also listed as Amber under the Birds of Conservation Concern 4 in the United Kingdom (JNCC 2021). In Taiwan, the Little Tern populations were also reported in declining trend in recent years (NTU Biodiversity Center 2020), mainly caused by the human disturbances to their breeding sites since many of them were not designated as protected areas (Cheng 2007). The Little Tern is currently classified as the class II species (i.e., rare and valuable species) under the Wildlife Conservation Act Republic of China (Taiwan) and it is the only species from the family Laridae who has breeding records in mainland Taiwan (Lin and Pursner 2020). However, little is known about their phylogeny, genetic diversity and population structure and to date, only one population genetic study has been conducted for the Japanese populations (Hayakaawa et al. 2022). According to the results from this recent study, two Little Tern populations in Japan (mainland Japan vs. Okinawa) are delimitated based on the analyses of molecular and morphological data but the factors that caused the population differentiation are still unclear. Long-term monitoring surveys are essential tool for the conservation and have been conducted

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continuously for the main Little Tern colonies in Taiwan. Whereas understanding on population connectivity for the protected species can provide valuable information that aid in constructing conservation strategies on how to manage the protection units, such the information in the Little Terns in Taiwan is currently unknown.

Compared with other tern species, the Little Tern is a small and slender species, weighted at about 49 to 53 g with body size of 20 to 28 cm long (Higgins and Davies 1996). This species has a long tail characterized by a deep fork and its upperparts are pale grey while underparts are white (Higgins and Davies 1996). They prefer to breed in colonies near coastal shallow water areas with sandy sediments (Lopes et al. 2015) but urban and/or industrial developments in and nearby these sites have made them susceptible to the habitat change or loss. Nevertheless, they were found to breed in alternative sites, for example salinas (Catry et al. 2004) and modified rooftops (Fujita et al. 2009) in response to the habitat changes. Therefore, the Little Tern may not exhibit high site fidelity like most seabirds because terns can exhibit high dispersal rates and are able to colonize new sites quickly when the quality of their habitat has decreased (Seward et al. 2018).

Although the taxonomy of tern species requires more investigations for the clarification, the Little Tern is currently known to represent three subspecies, which are the nominated populations, *S. a. albifrons* occurring in Europe, south to North Africa and west to western Asia, *S. a. guineae* being restricted to western and central Africa, and *S. a. sinensis* distributed along northern, western and eastern coasts of Australia, New Guinea and eastern Asia including Taiwan and Japan (Gill et al. 2022; Higgins and Davies 1996). Taiwan is located in the East Asian-Australasian Flyway travelled along by the subspecies *S. a. sinensis* so the Little Terns found in Taiwan are expected to be the *S. a. sinensis*. The Little Terns in Taiwan have been recorded to depart from their wintering

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grounds in April, start to breed in May and leave in August from Taiwan (Hung, 2009). They have been spotted to breed since early May in some sites located in Yilan, Hualien, Taoyuan, Hsinchu, Zhanghua, and Jiayi counties and Penghu archipelagos (NTU Biodiversity Center 2020).

The distribution of breeding populations for many seabird species may be shaped by their wintering distributions and migratory routes (Szczys et al. 2017). The main migratory routes can usually be divided into migrations along western coast and along eastern coast of a particular area (Dayton et al. 2017; Kralj et al. 2020). Dayton et al. (2017) proved that the European Whiskered Tern was differentiated into western and eastern subpopulations, each subpopulation was characterized with different migratory routes and wintering sites. For the Little Tern populations in Japan, only the populations from mainland Japan (Tokyo and Chiba) were known to travel to eastern Australia for wintering while the wintering information for other breeding colonies such as in Fukuoka and Okinawa are still investigating (Hayakawa et al. 2022). On the other hand, the migratory routes of the Little Tern in Taiwan also remain unclear but previous geolocator data collected has revealed the Little Tern from eastern coast of Taiwan (Yilan) has a short stopover in Philippines during the period between the end of July and middle of September (Chang et al. 2013). Later, it travelled to western part of Australia from September to January. It returned back and arrived to Taiwan in April with a stopover in Indonesia during February. Besides, the Little Tern in eastern Taiwan (Yilan) has been proved to travel to Broome, western Australia during its non-breeding season based on some recoveries of ringed bird records (Clare 2015).

Microsatellites markers are often used in the studies of population genetics in the past but there are many limitations such as time-consuming for the development, costly and less precise results obtained (Crates et al. 2019; Thrasher et al. 2018; Zimmerman et

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al. 2020). The technology for alternative genotyping approaches has received a great attention and been in progress. The genotyping by sequencing (GBS) approaches such as restriction site associated DNA sequencing (RADseq) methods (e.g., ddRADseq; Peterson et al. 2012) which can generate a large amount of genome-wide single nucleotide polymorphism (SNPs) data using the reduced representation genomic sequencing method has gained its advantageous reputation in studying population genetics from variety of living organisms with relatively low cost (Andrews et al. 2016; Peterson et al. 2012; Walters and Schwartz 2020). On the other hand, some studies have indicated that application of SNP data performed better than microsatellite data in detecting the population structure in the studying taxa. For example, a greater number of SNPs may be revealed by RAD sequencing which can increase the power to detect the population structure more accurately (Sunde et al. 2020; Zimmerman et al. 2020). However, the results could be biased if the sex-related pitfalls were not considered. Faux et al. (2020) pointed out that artificial effect on interindividual differences and population structure were present in GBS data of the three examined aquatic bird species (*Sula leucogaster*, *Sula dactylatra* and *Mergus octosetaceus*) due to a disparity of sizes of sequence reads between avian sex chromosomes (Z and W).

In this study, I firstly applied a standard DNA barcoding procedure using the mitochondrial (mt) Cytochrome c oxidase I (*COI*) gene sequence information extracted from the Little Tern populations in Taiwan to verify the subspecies status. Then, I evaluated the genetic diversity and population structure of the Little Tern populations in Taiwan using two different types of data: mtDNA sequences in control region (D-loop) and SNP data obtained from ddRADseq. This study also aimed at determining whether or not the Little Tern populations in western side of Taiwan differentiate from those in eastern side of Taiwan with respect to their putative migrations along the coasts of both

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sides. Additionally, since Taiwan is close to Japan and the Little Tern in both countries should belong to the same subspecies and may come from the same origin, the genetic information of the Little Tern populations in Taiwan and Japan was further explored by incorporating the mtDNA control region sequences from mainland Japan and Okinawa individuals available in the NCBI GenBank to evaluate their population connectivity as well as phylogeographic pattern.

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Chapter 2 Materials and Methods

2.1 Sampling and DNA extraction

Feather samples from 59 Little Tern chicks (36 and 23 in 2021 and 2022, respectively) were collected between May and July in 2021 and 2022. Sampling was conducted at four different breeding colonies (hereafter referred as populations) of this species in Taiwan, two from western side of Taiwan and another two from eastern side of Taiwan (Figure 2.1, Table 2.1). The sampling was limited to young-of-year fledglings at the breeding area to ensure the tissues were representative of local breeding sites. Each colony was visited at least once between May and July (breeding period) of the year. The chicks were hand captured within the breeding colony and the feather samples were obtained by pulling eight to twelve down feathers from each individual bird. Each feather samples were kept at room temperature in individual glassine envelopes separately. All chicks were released within ten to fifteen minutes of capture back to the chick creche. The duration of each visit to a colony was limited within 2 hours. Besides, I sampled one chick per nest to prevent sampling siblings. The sampling was performed under the permissions from the consent authorities (Appendix I and II) and the animal operations were approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University (Appendix III and IV). The genomic DNA was extracted using standard QIAamp DNA Micro kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's protocols with slight modifications adapted to the samples. The quantity and quality of each sample were tested using 1% agarose gel (Omega Bio-Tek, Norcross, GA, United States) electrophoresis and on a Nanodrop spectrophotometer. When handling and preparing the feathers, the possible risk of cross-contamination was carefully avoided by using a separate surgical blade for each sample.

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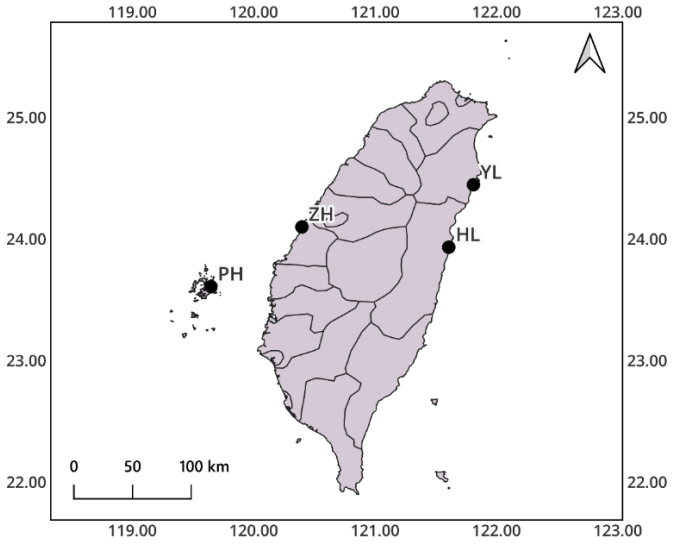

Figure 2.1 Map of Taiwan with the sampling localities for the Little Terns (*Sternula albifrons*) (PH, Penghu; ZH, Zhanghua; YL, Yilan; Hualien, HL) created using QGIS v3.28.0.

Table 2.1 Location and number of individual feather samples of the Little Tern taken (N) for genetic analyses per site in this study.

Group	Population	Site	N
West	Penghu (PH)	23°36’24’’N, 119°38’54’’E	16
West	Zhanghua (ZH)	24° 05’52’’N, 120°23’52’’E	11
East	Yilan (YL)	24° 26’43’’N, 121°48’41’’E	23
East	Hualien (HL)	23° 55’53’’N, 121°36’28’’E	9

2.2 Sequencing analysis

2.2.1 *COI* sequences

A DNA fragment with about 591 nucleotides from *COI* gene region were amplified by the polymerase chain reaction (PCR) using one pair of bird-specific *COI* primers: BirdF1 (5'-TTCTCCAACCACAAAGACATTGGCAC-3') and BirdR1 (5'-ACGTGGGAGATA-ATTCCAAATCCTG-3') (Hebert et al. 2004) and the EmeraldAmp Max HS PCR Master Mix (TaKaRa Bio Inc.). The PCR reactions were performed in 15 μl volumes on a Veriti™ Dx 96-Well Fast Thermal Cycler (Applied Biosystems) using 1.5 μl extracted DNA, 7.5 μl of PCR Master Mix 2X and a final concentration of 10 μM for each primer. The amplification regime consisted of 5 min at 95 °C followed by 35

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cycles with denaturation for 40 s at 95 °C, annealing for 30 s at 51 °C and extension for 40 s at 72 °C, followed in turn by a final 7 min at 72 °C. The PCR products were checked in a 1% agarose gel electrophoresis or using Qiaxcel Advanced System (Qiagen, Germany) and the successful ones were purified with AMpure XP beads (Beckman Coulter, Inc.) according to the manufacturer's instructions. The purified amplicons were then sent to Genomics BioSci and Tech (Taipei) for Sanger sequencing and either the forward or reverse direction of the *COI* gene fragment was sequenced with the same respective forward or reverse primer used in PCR reaction. A total of seven individuals failed from amplifying *COI* sequences were excluded from the downstream analyses.

2.2.2 mtDNA control region sequences

For obtaining the targeted sequences from the samples collected in this study, the variation in the 5' and 3' ends of the two overlapping fragments spanning the mtDNA control region was first assayed using the newly designed primer set DLF (5'-CCTAYACCCACCCATGACATTT-3') and DLR (5'-CTGTCGTTGACGTGTAAC-AAAGA-3'). The alternate primer DLR2 (5'-GCCGCGATTAAGAAAGGAA-3') was also designed and used for sequencing in the cases where the resulting sequences obtained using forward primer DLF contained too many ambiguous sites due to sequencing problem. For those cases, the fragments amplified using DLR2 primer were collated with DLF and DLR to generate a more complete sequence covering the control region of mitochondrial DNA. The PCR reactions were performed in 15 μl volumes on a Veriti™ Dx 96-Well Fast Thermal Cycler (Applied Biosystems) using 1.5 μl DNA extract, 7.5 μl of PCR Master Mix 2X and a final concentration of 10 μM for each primer. The amplification regime consisted of 4 min at 94 °C followed by 35 cycles with denaturation for 40 s at 94 °C, annealing for 40 s at 58 °C and extension for 1 min 15 s at 72 °C,

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followed in turn by a final 7 min at 72 °C. The PCR products were detected in a 1% agarose gel electrophoresis or using Qiaxcel Advanced System (Qiagen, Germany) and purified with AMpure XP beads (Beckman Coulter, Inc.) according to the manufacturer's instructions. The PCR amplicons were then sent to Genomics BioSci and Tech (Taipei) for Sanger sequencing. Then, the sequences from the two or three fragments were assembled and analysed for each individual.

2.2.3 ddRAD sequencing data

This study generates SNP data using ddRAD-based approach with a protocol modified from Peterson et al. (2012). The quality of each sample was first assessed using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) before preparing the ddRAD library. All of the qualified DNA samples were standardized to at least 23 ng/μl and were then digested with the restriction enzymes (EcoR1 and MspI) to produce small DNA fragments. I purified DNA using 1.5x volumes of AMpure XP beads (Beckman Coulter, Inc.) to remove the impurities after digestion. The sticky ends of the fragments were ligated with custom-designed adapters and the ligation products were size-selected (200-700 bp size range) using Ampure XP beads. The size-selected DNA were indexed with Nextera ® XT Index Kit by performing PCR. The products were purified using 0.8x volumes of AMpure XP beads and quantitated with a Qubit 3.0 fluorometer (Life Technologies). The qualified barcoded samples were pooled with equal quantity to create a library and sent to Genomics BioSci and Tech (Taipei) for sequencing using a MiSeq Reagent Kit v3 (2 x 300 bp) on Illumina MiSeq flowcell. After the quality of raw fastq files were assessed using FastQC v0.10.1 (Andrew 2010), the adapter sequences were removed using Trimmomatic v.0.36 (Bolger et al. 2014) with the default settings. Sequences were not filtered or trimmed for low quality.

doi:10.6342/NTU202300368

2.2.4 Sex determination

In order to assess the potential biases related to sex in ddRAD data analyses, the sex for each sample was determined by PCR amplification of CHD gene using one pair of specific primers: 2550F (5'-GTTACTGATTCGTCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTG-CTT-G-3') (Fridolfsson and Ellegren 1999) and the EmeraldAmp Max HS PCR Master Mix (TaKaRa Bio Inc.). The PCR reactions were performed in 12.5 μl volumes on a Veriti™ Dx 96-Well Fast Thermal Cycler (Applied Biosystems) using 1.0 μl extracted DNA, 6.5 μl of PCR Master Mix 2X and a final concentration of 10 μM for each primer. The amplification regime consisted of 5 min at 95 °C followed by 35 cycles with denaturation for 45 s at 95 °C, annealing for 45 s at 53 °C and extension for 45 s at 72 °C, followed in turn by a final 5 min at 72 °C. The PCR products were run in a 2% agarose gel electrophoresis and visualized in UV light. The samples that present with one band were identified as male while the samples present with two bands were identified as females. A chi-square ($\chi 2$) test with 95% confidence interval was conducted using Microsoft Excel to test whether the sex ratio of the samples is deviated from 1:1.

2.3 Data Analysis

2.3.1 *COI* sequences

Obtained sequences were checked, edited and aligned using ClustalW (Thompson et al. 1994) which with default parameters in MEGA-X v10.1 (Kumar et al. 2018). All of the sequences generated in this study were aligned with 31 *COI* sequences of Little Tern and 17 *COI* sequences from other *Sternula* species available in NCBI GenBank (Table S1) to confirm the field identification of Little Tern chicks and to verify if Little Terns sampled in Taiwan belong to the subspecies *S. a. sinensis* based on the classification

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scheme of Higgins and Davies (1996). The compiled and aligned sequences were then used to perform the phylogenetic analyses. The inferred phylogenetic tree was rooted by a sequence of an atypical black-capped tern species, *Phaetusa simplex* (GenBank accession: FJ028004) and a sequence of a noddy species, *Anous stolidus* (GenBank accession: DQ433312). The *COI* analyses were conducted by using maximum likelihood (ML) method as implemented in the RAxML 7.2.8 (Stamatakis et al. 2014) using the raxmlGUI 2.0 software (Silvestro and Michalak 2012). A general time-reversible (GTR) substitution model, with a gamma distribution that models rate variation across sites, was used for ML analyses. The branching supports of the inferred ML tree were evaluated using ML bootstrapping (Felsenstein 1985) with 1000 replicates performed. The ML tree reconstructed was visualized using FigTree v1.4.2 (Rambaut 2014). The branching supports can be considered robust if their bootstrap (BS) values are superior to 74% for ML analysis (Erixon et al. 2003).

2.3.2 mtDNA control region sequences

Genetic diversity

Two datasets were compiled for the downstream analyses, one contained only sequences from Taiwanese samples while the other contained sequences from both Taiwanese and Japanese samples. Those Japanese sequences were retrieved from NCBI GenBank (GenBank accessions: LC613038-LC613084) and included the sequences from samples located in mainland Japan at Fukuoka (FKK), Tokyo (TKY) and Chiba (CHB), and Okinawa islands (OKN). The compiled sequences were checked, edited and aligned with ClustalW (Thompson et al. 1994) with default parameters setting using the tools as implemented in the MEGA-X v10.1 package (Kumar et al. 2018). For obtaining the complete control region sequences, DLR2 was used as alternate primer for amplification

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and sequencing to overcome the issue of a consistent existence of approximately 65-bp long ambiguous bases occurred at around 500 bp from the 5' end of the control region in some samples when the original set of primers was used for sequencing. If the ambiguous bases still persisted after all, those bases were designated as 'N' or missing data.

DNA sequence polymorphisms including the number of haplotypes (*h*), number of segregating sites (*S*), haplotype diversity (H_d), and nucleotide diversity (π) values for each population, each group of populations and as overall in the dataset were estimated using DnaSP v5.0 (Librado and Rozas 2009). The haplotype networks were constructed and visualized with the PopART 1.7 (Leigh and Bryant 2015) using the median-joining network inference method (Bandelt et al. 1999).

Population structure

For examining Taiwanese populations, the dataset contained only samples of Taiwan was grouped accroding to the geographic regions (i.e. western and eastern Taiwan). For comparing genetic information of Taiwanese with Japanese populations, the dataset contained the samples from both countries was tested for different hierachical groupings (Table 3.2) to define the most suitable grouping for analyses since the degree of differentiation between Taiwanese and Japanese populations is unknown. All datasets were used to conduct the analyses described below in Arlequin (version 3.0; Excoffier et al. 2005). An analysis of molecular variance (AMOVA; Excoffier et al. 1992) was conducted to calculate the Φ_{ST} statistics using Kimura two-parameter distances with parameter (α) of 0.42 of the gamma distribution (Marshall and Baker 1997) and to examine the proportion of genetic variance explained by the differences in the dataset. For the interpretation of Φ_{ST}, Wright (1978) stated that the range 0.00 to 0.05 represents little, 0.05 to 0.15 represents moderate, 0.15 to 0.25 represents great and above 0.25 represents very great population differentiation. The division of populations in the dataset

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contained Taiwanese and Japanese populations was identified by the grouping with the highest percent of variation among groups (Φ_{CT}) as recommended by Dupanloup et al. (2002). The significance of the analyses was assessed based on 10,000 permutations coupled with p-value adjusted using false discovery rate (FDR) method (Benjamini and Hochberg 1995) to control the error rate. To estimate demographic history, Tajima's *D* and Fu's F_S tests were conducted in Arlequin to examine neutrality of the samples. Isolation by distance pattern was tested for the dataset by plotting a linear regression showing the relationship between the genetic (pairwise Φ_{ST}) distance and geographical distance among the populations. The dataset was also tested for isolation by distance (Rousset 1997) among the populations by using a Mantel test (Mantel 1967) with 10000 permutations implemented in Arlequin. The genetic distance matrix was built using the pairwise Φ_{ST} obtained from AMOVA while the geographical distance matrix was built by measuring the shortest potential travelling distances between sampling sites in Google Earth 6.2.1.

2.3.3 ddRAD sequencing data

SNP calling and filtering

The SNP calling and filtering tasks were processed using ipyrad v.0.9.84 (Eaton and Overcast 2020). First, the sequencing data was screened to remove individuals with low raw reads (< 100,000). The sequence reads were assembled using de novo method due to absent of published genome of Little Tern or any close relative as reference genome. According to Miller et al. (2007), doing high-throughput analysis will facing trade-off problem between the number of SNPs obtained and the number of missing data per locus. Therefore, different parameter settings were carried out for calling different SNP datasets. Since the main parameters that altered in various studies (Eaton and Ree 2013; Hudson

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et al. 2020; Suchan et al. 2017) were the similarity of sequences for clustering and the minimum number of samples per locus, so these parameters were altered to produce different SNP datasets and the number of recovered SNPs from the datasets (Table S2-S4) were compared to define the most suitable SNP dataset for downstream analyses. The final dataset was set to have 90% clustering threshold level for the sequence assembly, containing loci with a minimum number of 32 samples per locus and less than 50% missing sites. The samples were divided into two groups representing western and eastern sides of Taiwan (hereafter referred as two groups of populations), which the eastern side Taiwan containing YL and HL populations while western side Taiwan containing PH and ZH populations for the population genetic analyses. The downstream analyses were all conducted using R v.4.2.1 (R Development Core Team 2015) unless otherwise stated.

Genetic diversity

The pairwise population summary statistics including the heterozygosity (H_O and H_E), rarefied allelic richness (A_R) and inbreeding coefficient (F_{IS}) were calculated for each group and population using *hierfstat* (Goudet 2005) and *adegenet* v2.1.1 (Jombart et al. 2020). The missing data was replaced with mean allele values before the calculation using 'missingno' function in *poppr* v2.8 (Kamvar et al. 2018). One-way ANOVA was performed to test whether there are significant differences in mean H_O, H_E and A_R between the two groups and among the four populations using the basic statistical functions in R.

Population structure

The population structure was assessed using STRUCTURE v2.3.4 (Pritchard et al. 2000), discriminant analysis of principal components (DAPC) and principal component analysis (PCA) using 5113 SNPs after the SNP filtering. The analysis in STRUCTURE was run using admixture model and the number of genetic clusters (K) was set from 1 to

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5 with 10 iterations per K value. A burn-in of 500000 generations and 100000 MCMC chains were implemented for each iteration. The number of most appropriate clusters was determined using Evanno method (Evanno et al. 2005) in the STRUCTURE Harvester (Earl and Vonholdt 2012). The expected cluster membership for each individual was displayed using CLUMPP (Jakobsson and Rosenberg 2007) and DISTRUCT (Rosenberg 2004) according to the STRUCTURE results. The discriminant analysis principal component (DAPC) and principal component analysis (PCA) was implemented using package *adegenet* v2.1.1 (Jombart et al. 2020) and *ade4* (Dray and Dufour 2007) to further examine the population subdivision. The number of clusters in the dataset was first inferred using 'find.clusters' function without *a-priori* grouping information. The K-means clustering of principal components used were set from K = 1 to K = 5 and the optimal number of genetic clusters was assessed using Bayesian Information Criteria (BIC) which the optimal K would be associated with the lowest BIC value (Carlen and Munshi-South 2020). However, the subsequent analyses were conducted based on the *a-priori* clusters on the sampling locations (i.e., 2, represented by western and eastern sides of Taiwan). The number of principal components (PCs) retained was determined using optimal a-score ('optim.a.score' function) and cross-validation per Jombart ('xvalDapc' function). The population structure was explored in principal component analysis (PCA) to assess possible clusters among individuals. The PCA was plotted for only the first two principal components.

The pairwise F_{ST} estimates between the populations were calculated based on Weir and Cockerham method with 1000 permutations to estimate 95% confidence intervals and the *p*-values were corrected for false discovery rate (FDR) using *stAMPP* v1.5.1 (Pembleton et al. 2013). Isolation by distance pattern was tested for the dataset by plotting a linear regression showing the relationship between the genetic (pairwise F_{ST})

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distance and geographical distance among the populations. The isolation by distance pattern was also assessed by performing Mantel test based on 1000 permutations using 'mantel' function in *ecodist* (Goslee and Urban 2007). The genetic distance matrix was built using Prevosti's distance which can handle the missing data in the dataset while the geographical distance matrix was built by measuring the shortest potential travelling distances between the sampling sites in Google Earth 6.2.1. This study ran the analysis of molecular variance (AMOVA) to obtain the proportion of total molecular variance that explained the differentiation in the populations between western and eastern sides of Taiwan using *poppr*.

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Chapter 3 Results

3.1 *COI* sequences

3.1.1 Sequencing data

Among a total of 59 chick samples collected in the field, seven were failed on generating *COI* sequences during the laboratory work. Only 52 *COI* sequences were included in the subsequent phylogenetic analysis. The BLAST search results showed that the obtained *COI* sequences highly matched (> 98%) to the *COI* sequences of the *Sternula albifrons* deposited in GenBank except for one sequence (NA050_albifrons_Taiwan; sample collected from YL). This particular sequence highly matched (99.28%) to *Sternula antillarum* (GenBank accession: EU525523), a congeneric species of *S. albifrons*.

3.1.2 Phylogenetic analysis

The compiled *COI* dataset included 52 newly generated Little Tern *COI* sequences from four populations in Taiwan, 50 published *COI* sequences from four tern species in genus *Sternula* and two *COI* sequences from outgroup taxa. The length of the aligned *COI* sequences after trimming at 5' and 3'end sides of the sequences was 591-bp. 50 variable sites are present in this dataset with exclusive of outgroup sequences, among which 45 are parsimony-informative.

The Figure 3.1 showed the phylogenetic tree of *Sternula* inferred by maximum likelihood method. Two main clades were resolved with moderate and high bootstrap support values with each includes two sampled *Sternula* species. The clade I contained *S. albifrons* and *S. nereis* while clade II contained *S. superciliaris* and *S. antillarum*. All the species except '*S. antillarum*' examined were confirmed to be monophyletic. Actually,

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one of the collected '*S. albifrons*' chicks, NA050_albifrons_Taiwan, from Yilan (see above), was found to be nested within *S. antillarum* instead of *S. albifrons*. Within the clade of *S. albifrons*, the *S. albifrons* sequences were further divided into two groups, each with well-supported value (BS >82%): the subgroup I included the individuals from *S. a. albifrons* (samples from Europe, East Africa, western Asia) except one from Japan, the subgroup II contained all individuals from *S. a. sinensis* (samples from Australia, Japan, Korea and Taiwan). All the newly obtained sequences of *S. albifrons* from Taiwan except NA050_albifrons_Taiwan were clustered within the *S. a. sinensis* clade.

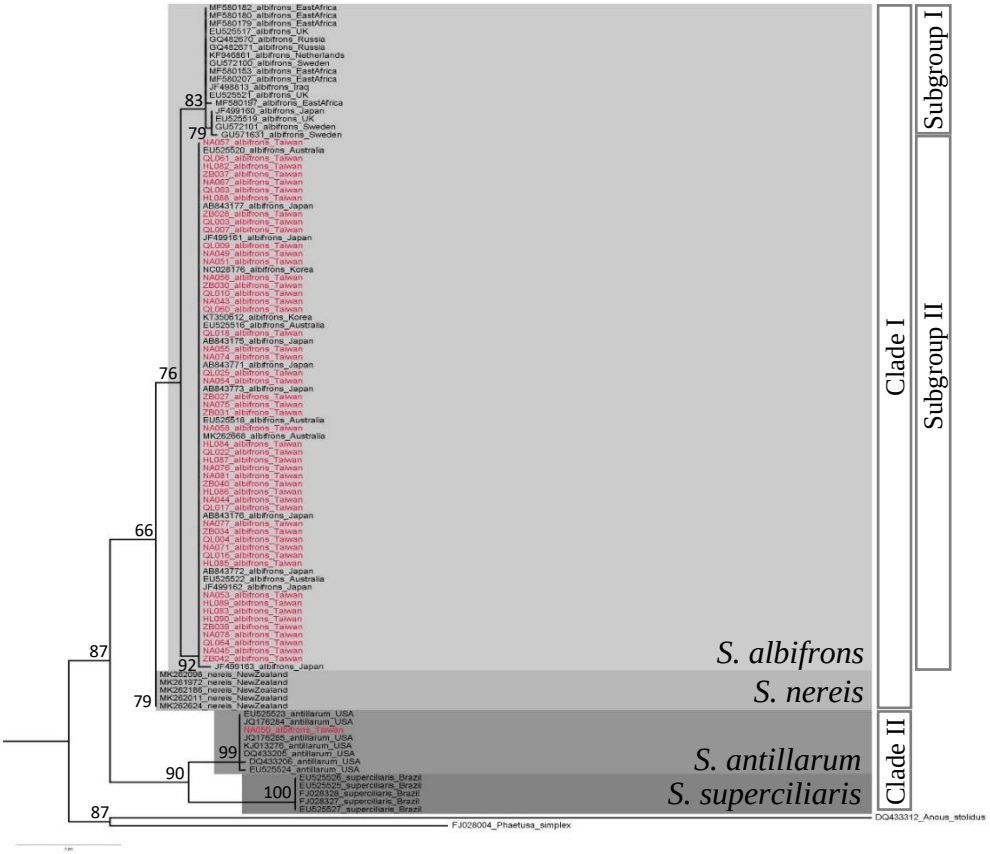

Figure 3.1 Phylogeny of *Sternula* reconstructed using Maximum Likelihood method with GTR+G model as implemented in RAxML based on *COI* sequences of genus *Sternula* retrieved from NCBI GenBank and newly obtained in this study (sample names in red). The tree was rooted by two outgroup taxa from *Phaetusa simplex* and *Anous stolidus*. Branch length is proportional to the inferred number of nucleotide substitutions. Numbers at the branches indicate the ML bootstrap support values.

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3.2 mtDNA control region sequences

3.2.1 Sequencing data

Since the identification of one individual collected from YL (NA050_albifrons_Taiwan) was confirmed to not be the S. *albifrons* species by DNA barcoding procedure, so the individual was removed from the subsequent population genetic analyses. Moreover, one individual resulted in bad quality in mtDNA control region sequencing was also excluded from the analyses. After sequence editing, alignment and trimming, a dataset of 861-bp long mtDNA control region sequences was constructed from 57 Taiwanese samples and 47 Japanese samples for downstream phylogeographic and population genetic analyses.

3.2.2 Genetic diversity

A total of 33 haplotypes with 28 polymorphic sites were identified from the mtDNA control region sequences of 57 Taiwanese samples in four populations (Table 3.1). Overall, all populations showed a high haplotype diversity. Yet, nucleotide diversity was relatively low, ranging from 0.005 to 0.007. The Tajima's D and Fu's F_S values were mostly negative which might indicate recent population expansion for all populations except for PH (D = 0.02, $p > 0.05$) (Table 3.1). However, only the Fu's F_S values in ZH and YL were significant, so such the implication should be taken with caution. The haplotype network constructed did not separate the samples into the two defined geographical groups (i.e., western and eastern sides of Taiwan) (Figure 3.2a). Six haplotypes were shared by the samples from more than one population (Hap_2, Hap_3, Hap_5, Hap_8, Hap_10, Hap_28). Of these, the most frequent haplotype was Hap_10 shared by seven individuals across PH, ZH and YL. Unique haplotypes are much abundant and often represented by a single individual.

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When the sequences from Taiwanese samples and Japanese samples were combined for the analyses, 53 haplotypes with 36 polymorphic sites were identified (Table 3.1). Similar to Taiwanese populations, the Japanese populations showed an overall high haplotype diversity but low nucleotide diversity in populations though the nucleotide diversity of Taiwanese populations was slightly but significantly higher than Japanese populations ($F = 9$, $p < 0.05$). The haplotype network constructed did not show a clear geographical assignment neither. All the haplotypes were tightly connected to each other with a maximum of three mutation steps for the connection between the neighbour haplotypes (Figure 3.2b). The three mostly common haplotypes were Hap_3, Hap_2, and Hap_10. Each is more or less predominated by the individuals from mainland Japan (Hap_3), Okinawa (Hap_2), and Taiwan (Hap_10), respectively.

Table 3.1 Genetic diversity estimated from the Little Tern populations in Taiwan and Japan based on mtDNA control region sequences.

Location	Pop	n	*h*	*S*	$H_d \pm$ SD	$\pi \pm$ SD	*D*	F_S
Taiwan		57	33	28	0.965 ± 0.011	0.005 ± 0.005		
	PH	15	9	14	0.924 ± 0.044	0.005 ± 0.005	0.02	-1.56
	ZH	11	9	15	0.945 ± 0.066	0.005 ± 0.004	-0.84	-3.42*
	YL	22	16	17	0.970 ± 0.022	0.005 ± 0.003	-0.57	-8.98*
	HL	9	8	17	0.972 ± 0.064	0.007 ± 0.006	-0.63	-2.49
Japan		47	24	19	0.917 ± 0.027	0.004 ± 0.003		
	FKK	7	5	8	0.857 ± 0.137	0.004 ± 0.002	-0.20	-0.61
	OKN	12	9	14	0.909 ± 0.079	0.004 ± 0.003	-1.02	-3.39*
	TKY	18	14	15	0.935 ± 0.052	0.004 ± 0.002	-1.13	-9.54*
	CHB	10	7	11	0.933 ± 0.062	0.004 ± 0.003	-0.43	-1.62
Mean			53	36	0.958 ± 0.010	0.005 ± 0.004		

Pop: populations; PH: Penghu; ZH: Zhanghua; YL: Yilan; HL: Hualien; FKK: Fukuoka; OKN: Okinawa; TKY: Tokyo; CHB: Chiba; n: sampling size; *h*: Number of haplotypes; *S*: Number of segregating sites; H_d: Haplotype diversity; π: Nucleotide diversity; SD: standard deviation; *D*: Tajima's *D*; F_S: Fu's F_S. *Asterisks denote significant values as follows: *p < 0.05.

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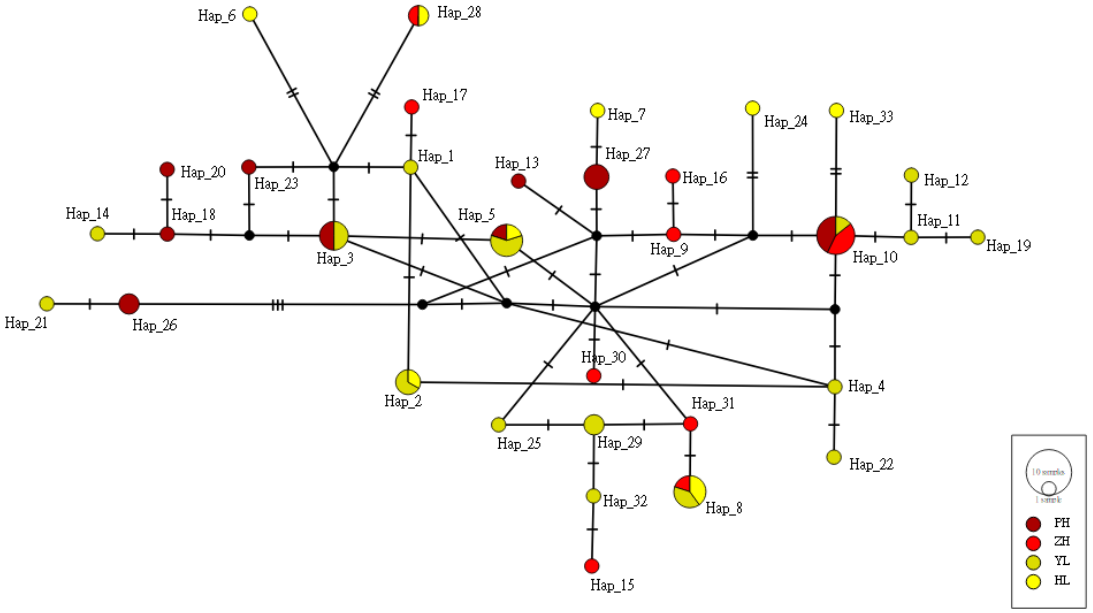
a)



b)

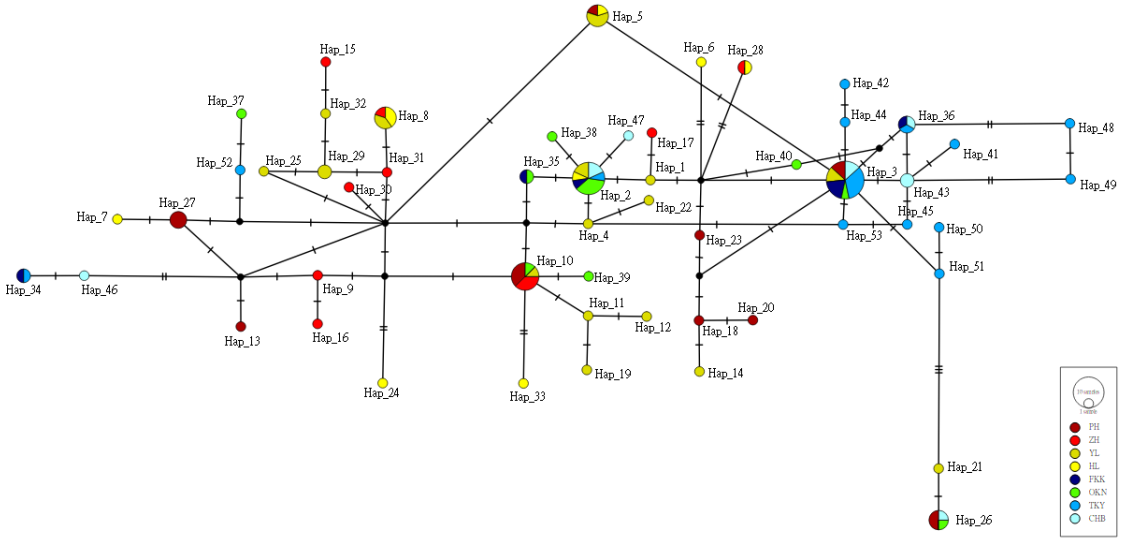


Figure 3.2 Median-joining haplotype network constructed from the Little Tern mtDNA control region sequences of (a) 57 Taiwanese samples; and (b) 57 Taiwanese combined with 47 Japanese samples. The black dots indicate the putative unsampled haplotypes. The size of each circle is proportional to the number of individuals with a particular haplotype. The dashes indicate mutational steps between two (sampled or unsampled) haplotypes. Colours indicate different populations (localities). Abbreviations: PH, Penghu; ZH, Zhanghua; YL, Yilan; HL, Hualien; FKK, Fukuoka; OKN, Okinawa; TKY, Tokyo; CHB, Chiba.

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3.2.3. Population structure

The AMOVA analysis (Table 3.2) showed no genetic differentiation for the samples between western and eastern sides of Taiwan (Φ_{CT} = -0.01, $p > 0.05$). The genetic variation was mostly explained by the variance within populations (99.41%). The pairwise Φ_{ST} values (ranged from -0.01 to 0.04) were low and non-significant for all pairwise comparisons in Taiwanese populations (Table 3.3). The correlation between geographical distance (km) vs. pairwise Φ_{ST} differences did not reveal any patterns of isolation by distance for the Taiwanese populations (Mantel test, r = -0.3315, $p > 0.05$, Figure 3.3a).

On the other hand, the AMOVA analyses based on the dataset containing Taiwanese and Japanese populations revealed that most variation still resided within populations but moderate differentiation among groups was observed, especially when three geographical regions (Taiwan, Okinawa, and mainland Japan) were partitioned (Figure 3.2). Moreover, the pairwise Φ_{ST} revealed moderate to very great ($\Phi_{ST} \geq 0.10$; Table 3.3) yet significant population differentiation in most of the pairwise comparisons between populations of Taiwan and Japan except for the pairwise comparisons of PH-FKK, YL-FKK, HL-FKK, HL-OKN, PH-CHB and HL-CHB (Table 3.2). The Φ_{ST} values obtained from the pairwise comparisons of ZH to all Japanese populations were particularly higher than any of other Taiwanese populations to Japanese populations. Among the Japanese populations including OKN, the Φ_{ST} values obtained from the pairwise comparisons of TKY to all Taiwan populations were generally higher than others. Finally, the Mantel test revealed a significantly positive correlation between geographical distance and pairwise Φ_{ST} differences for the dataset contained Taiwanese and Japanese populations (Mantel test, r = 0.630, $p < 0.05$, Figure 3.3b).

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Table 3.2 Results of analysis of molecular variance (AMOVA) for different groupings based on mtDNA control region sequences of the Little Tern populations in Taiwan and Japan.

Grouping	No. of groups	Source of variation	% of variance	Φ-statistics
west vs. east sides of Taiwan	2	Among groups	-1.01	Φ_{CT} = -0.01
		Among populations	1.60	Φ_{SC} = 0.02
		Within populations	99.41	Φ_{ST} = 0.01
Taiwan vs. Japan	2	Among groups	9.70	Φ_{CT} = 0.10*
		Among populations	3.92	Φ_{SC} = 0.04*
		Within populations	86.39	Φ_{ST} = 0.14*
Taiwan + Okinawa vs. Japan	2	Among groups	12.83	Φ_{CT} = 0.13*
		Among populations	2.64	Φ_{SC} = 0.03*
		Within populations	84.53	Φ_{ST} = 0.15*
Taiwan vs. Okinawa vs. mainland Japan	3	Among groups	14.20	Φ_{CT} = 0.14*
		Among populations	-0.03	Φ_{SC} = 0.00
		Within populations	85.82	Φ_{ST} = 0.14*

Φ_{CT}: the variance among groups relative to the total variance, Φ_{SC}: the variance among subpopulations within groups, Φ_{ST}: the variance among subpopulations relative to the total variance. *Asterisks denote significant values as follows: *$p < 0.05$.

Table 3.3 Pairwise Φ_{ST} (below diagonal) and geographic distance (km) (above diagonal) for the mtDNA control region sequences among the Little Tern populations in Taiwan and Japan.

Pop	PH	ZH	YL	HL	FKK	OKN	TKY	CHB
PH	-	93.76	421.72	489.42	1646.57	1049.69	2515.84	2647.21
ZH	0.04	-	327.96	395.66	1552.81	955.93	2422.08	2553.45
YL	0.03	0.00	-	67.70	1464.79	627.97	2247.49	2337.12
HL	-0.01	-0.04	-0.01	-	1532.49	695.67	2315.19	2404.82
FKK	0.05	0.17*	0.08	0.03	-	1006.83	1062.37	1115.45
OKN	0.13*	0.14*	0.10*	0.07	0.08	-	1638.39	1694.92
TKY	0.13*	0.28*	0.19*	0.17*	-0.03	0.25*	-	186.00
CHB	0.08	0.21*	0.12*	0.08	-0.11	0.09	0.01	-

Pop: populations; PH: Penghu; ZH: Zhanghua; YL: Yilan; HL: Hualien; FKK: Fukuoka; OKN: Okinawa; TKY: Tokyo; CHB: Chiba; *Asterisks denote significant values as follows: *$p < 0.05$.

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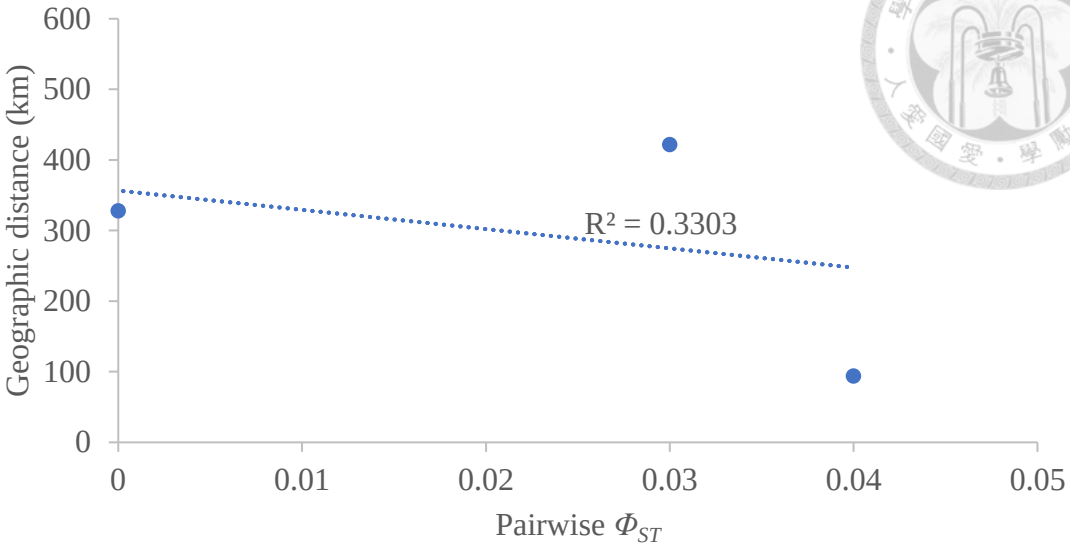
a)



b)

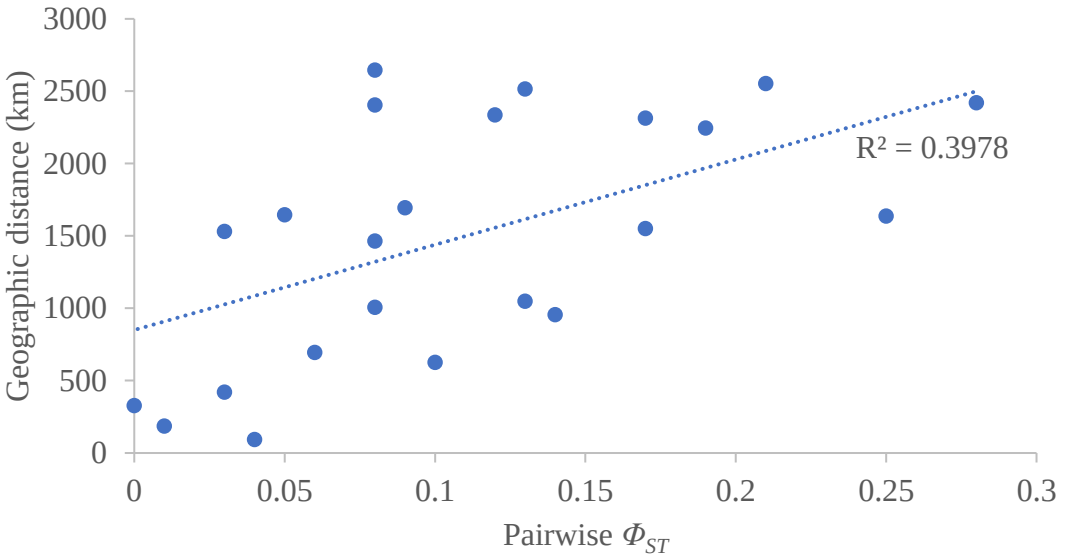


Figure 3.3 Correlation of genetic (pairwise Φ_{ST}) distance vs. geographical distance (km) for the mtDNA control region sequences of the Little Tern populations a) in Taiwan b) in Taiwan and Japan. Only positive pairwise Φ_{ST} values were presented.

3.3 ddRAD sequencing data

3.3.1 Sequencing data

A total of 12,242,379 raw reads was obtained from the ddRAD sequencing data, with an average of 244,847 reads per individual. The preliminary screening of sequencing data removed all individuals with low raw reads but retaining the individuals from HL

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population due to insufficient sampling size. Besides, the individual NA050_albifrons_Taiwan was excluded from the analyses due to wrong species identification. Therefore, the final dataset involved 50 individuals and the total SNPs retained was 5113, with a missing rate of 42.24% after the filtering process performed in ipyrad pipeline. Among the 50 individuals, 28 were females. Overall, the sex ratio of the sampled chicks was not significantly deviated from 1:1 in populations although high percent of females obtained in HL ($\chi^2 = 4.51$, $p > 0.05$, Table 3.4).

Table 3.4 Sex ratio of the Little Tern chick samples (N = 50) in Taiwanese breeding populations.

	PH	ZH	YL	HL
Number of females	8	5	8	8
Total	15	9	17	9
% of females	53	56	47	89

3.3.2 Genetic diversity

The number of polymorphic loci ranged from 7049 to 8211 among four populations. The populations from western side of Taiwan (9217) revealed a higher number of alleles than those from eastern side of Taiwan (8429) (Table 3.4). However, the rarefied allelic richness and mean observed and expected heterozygosity were higher in populations from eastern side of Taiwan (1.310, 0.40 and 0.31, respectively) than in those from western side of Taiwan (1.294, 0.35 and 0.29, respectively). There were no significant differences for the rarefied allelic richness (A_R), mean observed (H_O) and expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}) obtained between these two groups. The F_{IS} was negative for all populations, showed an absence of inbreeding. The H_O was higher than H_E in all populations in Taiwan, indicated an excess of heterozygosity, which further supported by negative F_{IS} values calculated.

doi:10.6342/NTU202300368

Table 3.5 Population genetic diversity statistics for the ddRAD sequencing data of the Little Tern populations in Taiwan.

Group	Population	n	N_A	A_R	H_O	H_E	F_{IS}
West		24	9217	1.294	0.35	0.29	-0.144
	PH	15	8211	1.305	0.37	0.30	-0.167
	ZH	9	7440	1.276	0.32	0.27	-0.137
East		26	8429	1.310	0.40	0.31	-0.236
	YL	17	7731	1.274	0.35	0.27	-0.190
	HL	9	7049	1.357	0.51	0.35	-0.414

PH: Penghu; ZH: Zhanghua; YL: Yilan; HL: Hualien; n: number of individuals sampled; N_A: number of alleles; A_R: allelic richness; H_O: observed heterozygosity; H_E: expected heterozygosity; F_{IS}: inbreeding coefficient.

3.3.3 Population structure

The pairwise F_{ST} differences between two geographical groups showed very little differentiation but statistically significant values (pairwise F_{ST} = 0.01). Besides, the pairwise F_{ST} estimates among the four populations were also very small (ranged from 0 to 0.07) but significant ($p < 0.05$) for all values (Table 3.5). The AMOVA result (Table 3.6) showed no genetic differentiation between two groups as the proportion of variance explained for the variation between groups was negative (-2.54%) and non-significant. Most of the variances were explained by the variation within populations (93.96%). The clustering analyses from DAPC (Figure 3.4), PCA (Figure 3.5) and STRUCTURE (Figure 3.6) did not reveal any population subdivision from the samples. Two groups of Little Tern populations were strongly overlapped to each other in the obtained PCA result. Moreover, DAPC with all PCs initially retained also suggested one cluster ($K = 1$) in the data. The correlation between geographical distance (km) vs pairwise F_{ST} differences did not reveal any patterns of isolation by distance for the data (Mantel test, $r = 0.5572$, $p > 0.05$, Figure 3.7).

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Table 3.6 Pairwise F_{ST} estimated (Weir & Cockerham method) (below diagonal) and geographic distance (km) (above diagonal) among the Little Tern populations in Taiwan based on ddRAD sequencing data.

Population	PH	ZH	YL	HL
PH	-	93.76	421.72	489.42
ZH	0.00*	-	327.96	395.66
YL	0.02*	0.01*	-	67.70
HL	0.03*	0.06*	0.07*	-

PH: Penghu; ZH: Zhanghua; YL: Yilan; HL: Hualien. *Asterisks denote significant values as follows: *$p < 0.05$

Table 3.7 AMOVA for the ddRAD sequencing data of the Little Tern populations in western and eastern sides of Taiwan.

Source of variation	Variance components	% of variance	F statistics
Among groups	-25.04	-2.54	F_{CT} = -0.03
Among populations	84.55	8.58*	F_{SC} = 0.08
Within populations	926.32	93.96*	F_{ST} = 0.06

Grouping: western vs. eastern side populations in Taiwan; F_{CT}: differentiation between groups; F_{SC}: differentiation among populations within groups; F_{ST}: differentiation within populations; *Asterisks denote significant values as follows: *$p < 0.05$.

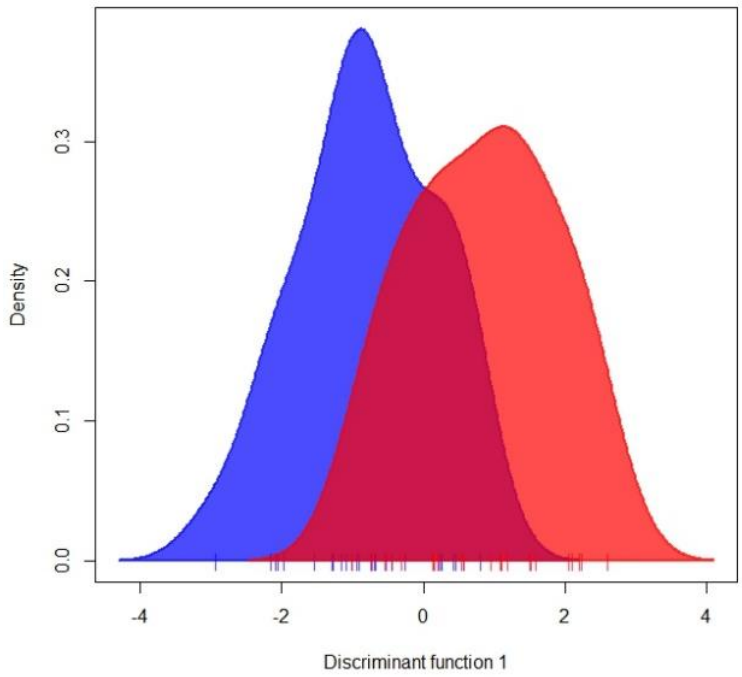

Figure 3.4 Density plot of Discriminant analysis of Principal Components (DAPC) for the ddRAD sequencing data of the Little Tern populations in Taiwan constructed based on *a priori* cluster information using the first discriminant function and 10 principal components. Vertical bars indicate individual assignments of Little Tern samples (n = 50) collected from western (red) and eastern (blue) sides of Taiwan. The individuals in different groups were represented by different colours.

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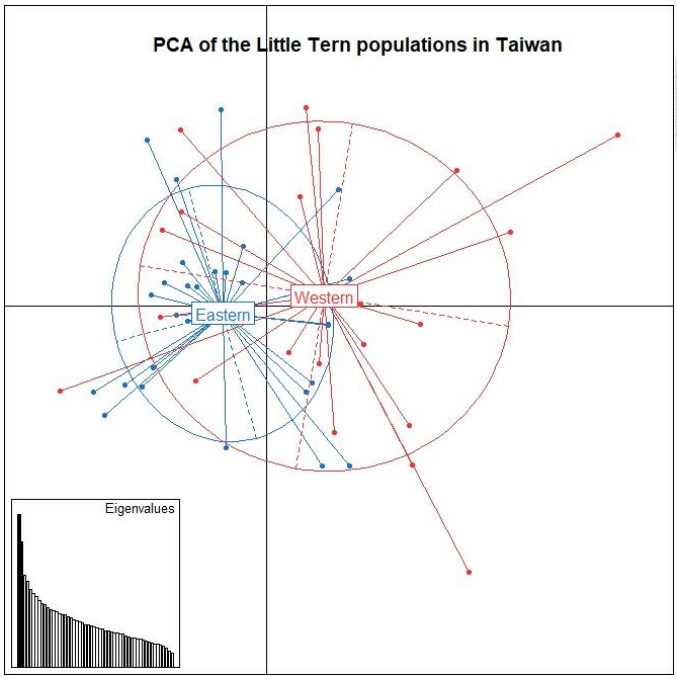

Figure 3.5 Principal Component Analysis (PCA) for the ddRAD sequencing data of Little Tern samples (n = 50) collected from western (red) and eastern (blue) sides of Taiwan. The individuals in different groups were represented by different colours. The PC1 explained 4.19% while PC2 explained 3.98%.

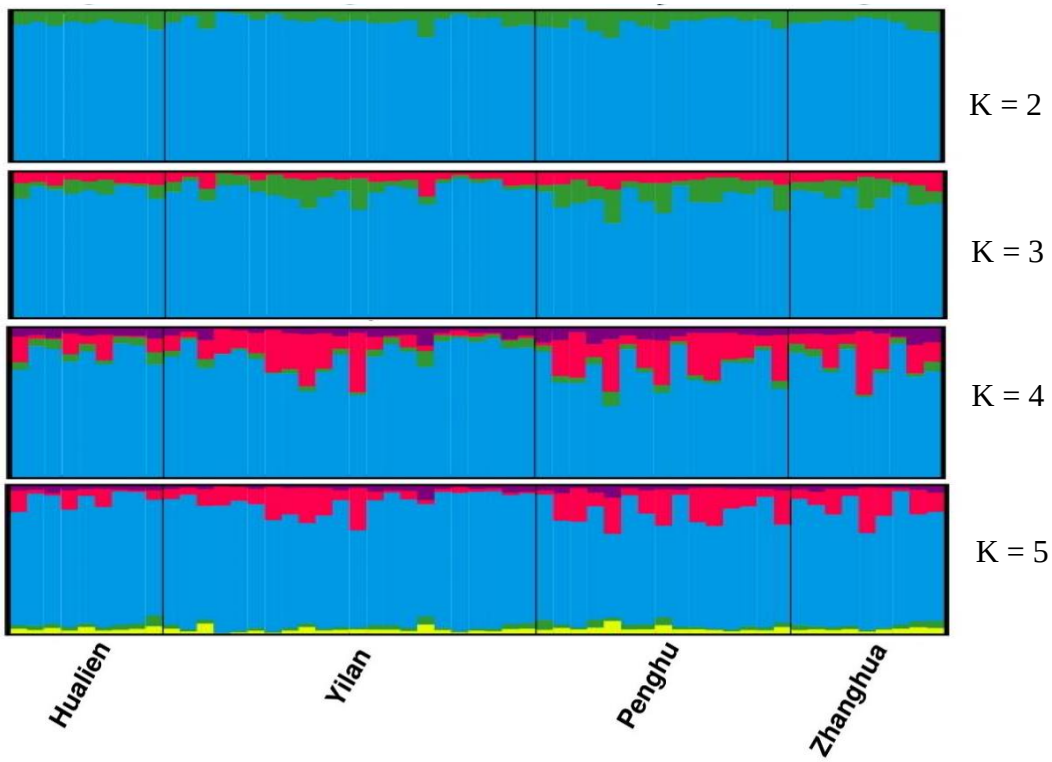

Figure 3.6 Results of clustering analysis for the ddRAD sequencing data of the Little Tern populations in Taiwan inferred by STRUCTURE for K = 2-5. Samples are grouped by sampling sites.

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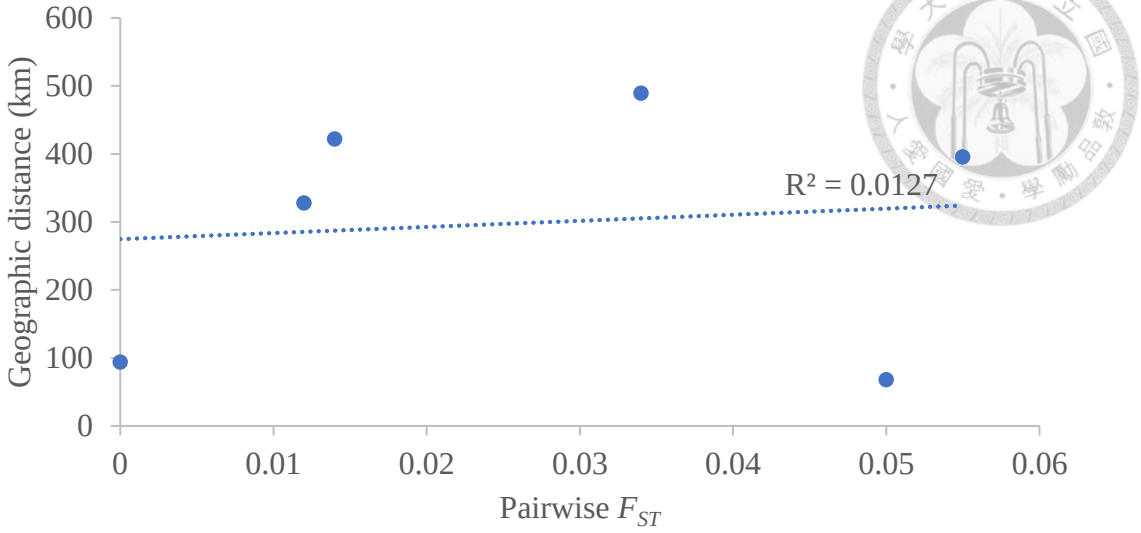

Figure 3.7 Correlation of genetic (pairwise F_{ST}) distance vs. geographical distance (km) for the ddRAD sequencing data of the Little Tern populations in Taiwan. Only positive pairwise F_{ST} values were presented.

3.3.4 Sex-biased artifact

To assess whether or not the estimation of genetic diversity and inference of population differentiation were sex-biased, the ddRAD sequencing data were further partitioned according to sex of the samples. Here, the populations contained less than five individuals from each sex were excluded due to small sample size for the accurate estimations. Thus, the downstream analyses were only conducted in PH and YL populations. The results showed that there was no significant difference for the estimated genetic diversity when only females or males were included for the analyses (Table 3.8). The pairwise F_{ST} estimated for both sexes were similar and both indicated no population differentiation (0.01 for both sexes; Table 3.9). Moreover, the result from PCA analysis (Appendix V, Figure S1) did not suggest neither a likely bias in estimation associated to the sex, i.e., individuals of the same sex had a propensity to cluster together, as shown in a previous study with GBS data (Faux et al 2020).

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Table 3.8 Comparison of genetic diversity statistics between female and male populations on ddRAD sequencing data in PH and YL populations.

Group	Population	Sex	n	N_A	A_R	H_O	H_E	F_{IS}
West	PH	Female	8	7135	1.250	0.30	0.25	-0.187
		Male	7	7118	1.236	0.27	0.23	-0.128
East	YL	Female	8	6812	1.218	0.27	0.21	-0.210
		Male	9	7000	1.219	0.27	0.22	-0.183

PH: Penghu; YL: Yilan; n: number of individuals sampled; N_A: number of alleles; A_R: allelic richness; H_O: observed heterozygosity; H_E: expected heterozygosity; F_{IS}: inbreeding coefficient.

Table 3.9 Pairwise F_{ST} estimated (Weir & Cockerham method) between females (above diagonal) and males (below diagonal) for the PH and YL populations based on ddRAD sequencing data.

Population	PH	YL
PH	-	0.01*
YL	0.01*	-

*Asterisks denote significant values as follows: *$p < 0.05$

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Chapter 4 Discussion

4.1 Taxonomic status of Little Terns in Taiwan

Present study confirms that Little Terns from different breeding sites in Taiwan are mostly the subspecies *S. a. sinensis*. This subspecies diverges genetically from *S. a. albifrons* (mean *p*-distance = 0.01 in *COI*). These two subspecies can also be distinguished by morphological differences. For instance, *S. a. albifrons* has the first primary with pale brown shaft whereas *S. a. sinensis* has very white shaft (Higgins and Davies 1996). Since Taiwan is located within the East Asian-Australasian flyways for *S. a. sinensis*, the result from this study is not so surprising. However, the present phylogenetic analysis uncovered that one individual (NA050_TWN_YL) collected from YL, Taiwan turns to be the Least Tern (*S. antillarum*), a congeneric species of the Little Tern and both species do not have overlapping breeding range. To our knowledge, the Least Tern only breed in the continents of North and South America (BirdLife International 2022) where no breeding records were reported for the Little Tern. Nevertheless, Pyle et al. (2001) found some small colonies (less than 10 individuals for each species) where *S. a. sinensis* and *S. antillarum* (subspecies unknown) co-existed and bred on the same site on Sand Island, Midway Atoll in the North Pacific during 1999 and 2000. This is the only confirmed record indicating that the Little Tern and Least Tern can share the same breeding area during the same breeding season. Since the morphology of both species are very similar, the Least Tern, particularly the younger breeders may be unable to differentiate their original populations from Little Tern populations and migrate with the Little Tern populations when both species have overlapping breeding area. On the other hand, migratory birds can get lost during migration due to some reasons. For example, a Black-browed Albatross originated from the South Atlantic was found in a

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breeding colony of the Northern Gannets in the North Atlantic (Farmer 2007). The albatross was believed to get lost on its migratory route due to a storm that blown the bird over the equator and deviated from its normal route. Therefore, another possible reason to explain the appearance of the Least Tern in Taiwan is that the parents of the chick are believed to get lost due to bad weather while migrating to their breeding grounds in America and somehow accidentally followed the Little Tern colonies to breed in Taiwan.

On the other hand, hybrids between closely-related species in terns were documented in the previous studies (Mostello et al. 2016; Yang et al. 2018). The particular sample NA050_TWN_YL is suspected of whether it is an offspring of Least Tern conspecific pair or Little/Least Tern mixed pair. In this study, the species identity was mainly confirmed by the maternally-inherited *COI* gene. Still, the PCA analysis based on ddRAD sequencing data separated the sample NA050_TWN_YL from the Little Tern samples, indicating non-hybrid origin of the sample NA050_TWN_YL (Appendix V, Figure S2). However, the STRUCTURE analysis was not able to partition the samples into two clusters (Appendix V, Figure S3). It is known that uneven sampling sizes between different groups (Little tern vs. Least Tern) may lead to inaccurate results inferred by the STRUCTURE (Puechmaille 2016). Nevertheless, continuous monitoring in breeding site YL as well as other sites in Taiwan should be conducted to confirm the presence of the Least Tern individuals and/or potential hybrids of the Little/Least Tern pair in Taiwan.

4.2 Genetic diversity

The present study is the first study of population genetic analyses of the Little Tern inferred from both mtDNA and genome-wide SNP markers which can improve the power of molecular data and resulted in complementary analyses. The mtDNA control

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region sequences and ddRAD sequencing data with 5113 SNPs retained were used to examine the population genetics of the Little Tern in Taiwan. Besides, the mtDNA control region sequences alone were used to further examine the extent the connectivity of the Little Tern populations among Taiwan, Okinawa and mainland Japan. The mtDNA control region sequences in the present study revealed high haplotype diversity as many individuals possess the unique haplotype by their own, but consistently low nucleotide diversity in the populations of the Little Tern, either in Taiwan or Japan. The result of high haplotype diversity (ranged from 0.92 to 0.97) in the Little Tern populations in Taiwan was mostly consistent with other migratory bird species inferred using the same type of molecular data, including the Least Tern populations in the U.S. (ranged from 0.42 to 0.92) (Draheim et al. 2010; Whittier et al. 2006) and Black-legged Kittiwake populations in the Pacific (ranged from 0.81 to 0.98) (Sauve et al. 2019). On the other hand, the gene diversity (H_E) in migratory seabird species reported ranged from 0.00 to 0.30 inferred by the SNP data (Byerly et al. 2022; Kersten et al. 2021; Lois et al. 2020, Perez et al. 2020). From these, the gene diversity of Little Tern populations in Taiwan (ranged from 0.32 to 0.52) was higher than other tern species, including the Elegant Tern from U.S. populations (ranged from 0.27 to 0.28) (Perez et al. 2020) and Roseate Tern (ranged from 0.16 to 0.22) from western Atlantic populations (Byerly et al. 2022). The higher value of gene diversity found in Little Tern populations in Taiwan may indicates higher degree of connectivity among populations than other tern populations mentioned above since higher genetic diversity was found in the populations with higher rates of gene flow (Gómez-Fernández et al. 2016). Negative Tajima *D* and Fu's *Fs* might indicate recent population expansion, but most of the values were not significant for the Little Tern populations in Taiwan. Besides, the evidences of recent population expansion including star-like pattern of haplotype network and low haplotype diversity were absent

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in this study. Therefore, there were insufficient evidences to conclude that the Little Tern populations in Taiwan were experiencing recent population expansion.

The genetic variation of a population describes the responses of a population to the changing environmental conditions and it is important to determine the fitness of the population in the environment (Abdul-Rahman et al. 2021). The patterns of genetic diversity of populations can be influenced by various factors and the individuals from populations with high genetic diversity can often adapt better to the changes in their environment. Some potential risks that could reduce dramatically the genetic diversity of the populations and should be noticed here. One of the factors that affect the genetic diversity is the intensity of predation pressure in the habitat as stated in the study of Bicknell et al. (2012). This study proved the Atlantic populations of Leach's Storm-Petrel with intense predation pressure to have low haplotype diversity (ranged from 0.51 to 0.78) while Pacific populations of the species with less intense predation pressure showed high haplotype diversity (ranged from 0.91 to 0.93). During the field work of this study, there were stray dogs being observed sometimes in the areas surrounding the sampling site of this study (e.g., in PH). These dogs are potential introduced predators to the local Little Terns. In PH, the footprints of dogs were found beside the predated eggs and are the evidence of nest disturbance in the breeding sites and of high mortality of chicks of the Little Tern during the breeding and/or hatching period of the birds in year 2021 (Lee 2021; Liu 2021; author, personal observation). Furthermore, the bad habitat quality can cause a loss of genetic diversity in the seabird populations, for example the Least Tern populations in the U.S. (Draheim et al. 2012). The large-scale habitat destruction caused the Least Tern populations declined and resulted in lower genetic diversity of contemporary populations compared to historical populations. The breeding ground of the Little Terns in Zhanghua (sampling site ZH) located in western coast of Taiwan has

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been almost destroyed (Figure 3.8) and the surrounding area has rapidly developed into industrial zone (Figure 3.9) since only a few years ago which would be a potential risk to exempt the site from the breeding populations in the future in Taiwan.

Although the haplotype diversity obtained from current Taiwanese Little Tern populations was generally high among the populations studied, but some cautions for the interpretation of the results should be taken. Sauve et al. (2019) suggested the current genetic variation may reflect the historical events combined with contemporary processes underwent by the populations if negative Tajima's *D* and Fu's *Fs* results were obtained which indicate the populations were not in equilibrium for mutation and genetic drift. The changes in genetics take time and the time needed is depending on various factors (Neigel and Avise 1986). The negative Tajima's *D* and Fu's *Fs* results (although only few values were significant) might indicate ongoing population growth in the Little Tern populations in Taiwan. However, the Little Tern has a long live span and the long-lived seabirds may retain their genetic signature over a long period after they suffered a bottleneck event, thus the interpretation of the results should be taken with caution (Lopez et al. 2020; Weiser er al. 2013).

Figure 3.8 An image captured during sampling in ZH showing the nesting ground of the Little Terns in ZH was limited due to the industrial planning.

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Figure 3.9 The sampling site in ZH has rapidly developed into industrial zone.

4.3 Population structure

The analyses based on mitochondrial and genome-wide SNP markers in the present study obtained consistent results to conclude that there is little to no population structure for the Little Tern in Taiwan. Based on the analyses of both types of data, the AMOVA did not reveal significant genetic differentiation from the populations between western and eastern sides of Taiwan, which further supported by the overall low pairwise F_{ST} values estimated. Such the absence of population structure may be resulted from absence of physical barriers to gene flow among or between the populations. The physical barrier, for example a very large area of land may isolate the seabird populations into western and eastern subpopulations (Friesen 2015) including the Caspian Tern populations in North America (Boutilier et al. 2014) and Eurasian Whiskered Tern populations in Europe (Dayton et al. 2017). In Taiwan, some terrestrial bird species, such as the Steere's Liocichla, displayed an east-west differentiation due to the existence of high mountain range as a biogeographical barrier that restricts the gene flow between populations in western and eastern sides of Taiwan (Peng 2006). However, the habitat characteristics as well as dispersal ability of terrestrial birds and seabirds are different and the mountain ranges may not be one of potential biogeographical barriers to constrain the

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gene flow in seabirds (e.g., Little Tern) since they utilise the coastal areas for breeding and foraging instead of terrestrial areas. The mixed haplotypes in the mtDNA network and clustering analyses based on ddRAD sequencing data that estimated one cluster (K = 1) in the samples supported the hypothesis of high gene flow among the four different populations in Taiwan. Furthermore, the geographical scale in the present study may be too small to detect the population differentiation since the published studies for seabirds that detect population differentiation were at a much larger scale. A study for another tern species (Elegant Tern) conducted at geographical scale larger than the present study had still found no significant differentiation between the populations in Mexico and Southern California (Perez et al. 2020), indicating the population connectivity is still high between populations in the studied area at that particular scale.

Low levels of philopatry in Little Terns may also contribute to weak population structure. Despite most seabirds exhibit high levels of philopatry, some tern species had been reported for weak philopatry, for example the Common Tern and Least Tern (Wernham et al. 2002). The species with weak philopatry do not simply return back to their natal breeding sites but are likely to choose other favourable sites to breed (Coulson 2016). Little Terns have been recorded to move their colonies to new sites in response to the habitat change (Catry et al. 2004; Huang 2015). For example, Little Terns in Japan inhabited along the coast have shifted their habitats from coastal to riverside areas after the construction of dam which caused erosion in the coastal areas (Huang 2015). Breeding in alternative sites have indicated that Little Terns exhibit weak philopatry and can quickly adapt to the changing environmental conditions and search for a more suitable place for breeding.

Site fidelity is the tendency to return to the same breeding sites or local population (Sandercock 2003). As a general rule, species with high site fidelity could decrease the

doi:10.6342/NTU202300368

rate of gene flow among the colonies and lead to population structuring. To date, no studies related to the site fidelity of the Little Tern have been conducted. The banding and resight data (Yilan bird society, unpublished data) revealed frequent interchange of colonies in Taiwan in different breeding years, which can be supported by the shared haplotypes (Hap_3, Hap_5, Hap_8, Hap_10 and Hap_28) across the populations and between western and eastern sides of Taiwan (Figure 3.2a). The breeding sites of the Little Tern in Taiwan are not necessary to be fixed as the birds often change their breeding locations across western and eastern sides of Taiwan in different breeding years. However, a few individuals were recorded on returning to the same locations (but not all returning to the same breeding sites) for breeding in the following years of the sampling release. The Least Tern, a congeneric species and exhibits similar life histories to the Little Tern, was proved to display low site fidelity and can disperse a long distance up to 300 to 1000 km from natal colonies (Renken and Smith 1995). Although the extent of breeding site fidelity of the Little Tern is still unclear, but the banding data supported that the Little Tern in Taiwan may exhibit low site fidelity as well and was known to disperse to other colony sites.

The dispersal ability may directly impact the degree of gene flow among populations and the patterns of dispersal can be influenced by the sex differences, site availability (Greenwood 1980), quality of habitats (Steiner and Gaston 2005), and stability of environmental conditions (Fagan et al. 2001). Moreover, the patterns of breeding dispersal in tern species can vary within year and can be caused by changes of nest site after the first nest attempt failed (Greenwood and Harvey 1982). In fact, the breeding phenology of tern species would associate with the availability of food resources around the breeding habitats and the dispersal may depend on the food availability. For example, breeding season of the Common Tern in New York were overlapped with a

doi:10.6342/NTU202300368

seasonal increase in prey abundance and food for terns peaked and began to decline before the period of peak demand of food by chicks (Safina et al. 1988). The Little Terns may move from western to eastern sides of Taiwan due to second nesting attempt that try to search another habitat with abundant food by moving along the coastline following the distribution of their preys. At this moment, the populations from western and eastern sides of Taiwan may be mixed together, resulting in panmixia among the populations and groups. On the other hand, the arrival time of Little Tern populations in PH and ZH was observed to be earlier than that of in YL and HL (Hung C-H, personal communication). This indicates that the arrival time of the birds may be in synchrony with the dynamics of their preys surrounding the waters of Taiwan. Little Terns prefer to feed on very small fish items, such as anchovies (Gochfeld et al. 2020). As stated by Chiu et al. (1997), the stock of anchovies migrated to the coastal waters in southwestern of Taiwan during March and extended north eastward during April and the abundance was peaked during May in the Yilan Bay, northeast of Taiwan. The tern individuals arrived earlier in southwestern Taiwan such as in PH and ZH; the timing may closely match with the peak of food availability in the regions during April. Similarly, the tern individuals arrived later in northeastern Taiwan; the timing may closely match with the peak of food availability during May in the Yilan Bay.

Another factor that may contribute to little genetic differentiation between the populations from western and eastern sides of Taiwan is that the presence of same non-breeding areas for Taiwanese Little Tern. Overlapping wintering sites can mix the birds from different breeding sites during non-breeding season and promote gene flow among the populations (Friesen 2015). Based on the geolocator studies (Chang et al. 2013), one Little Tern individual from YL was known to migrate to western Australia for wintering but the wintering grounds for Little Terns from western side of Taiwan is still unknown.

doi:10.6342/NTU202300368

Since there was no genetic differentiation between the populations in western and eastern sides of Taiwan, the Little Tern populations in western side of Taiwan are probably having overlapped wintering sites (i.e., western Australia) with eastern side populations. Furthermore, geolocator data showed the Little Tern individual migrated from YL to western Australia but came back to stay or cross along the western coastline of Taiwan (PH) and eastern coast of mainland China (Chang et al. 2013). This implies that the migratory routes of Taiwanese Little Terns may overlap in some extent between western and eastern sides at least in different breeding years.

In contrast, different migratory flyways and wintering sites may shaped the seabird populations. For instance, the Whiskered Tern in Europe exhibited strong population differentiation between the samples from western and eastern Europe due to different subpopulation-specific flyways and wintering sites of the birds which can isolate the populations during non-breeding seasons (Dayton et al. 2017). The AMOVA results based on mtDNA control region sequences from Taiwanese and Japanese Little Terns suggested a moderate to great differentiation among the populations from Taiwan, Okinawa and mainland Japan, supported by pairwise Φ_{ST} estimations among the three groups of populations. The haplotype network did not clearly separate the samples into three geographical groups but the first three most common haplotypes were prevalent for mainland Japan, Okinawa and Taiwan samples, respectively, showing the Little Tern populations are frequently connected but with some restrictions on gene flow that caused the differentiation among the three populations. The population structure detected may be due to different migratory flyways and wintering sites for the differentiated populations. Hayakawa et al. (2022) stated that low frequencies of gene flows may be occurred between Okinawa and mainland Japan populations continuously for a long time, suggesting the migratory routes and wintering sites of the Okinawa populations might be

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different from the mainland Japan populations. The Tokyo and Chiba populations were known to migrate to eastern Australia for wintering but the wintering site of Okinawa populations are currently unknown (Fujii et al. 2014). The haplotype network and pairwise Φ_{ST} differences from the dataset indicated that the Taiwan populations were more closely related to Okinawa population rather than mainland Japan populations, suggesting that the Okinawa population might be wintering in western Australia and the overlapping wintering sites with the Taiwan populations which promote the potential gene flow between Okinawa and Taiwan populations. According to Fujii et al. (2014), the Tokyo and Chiba populations stopover in Taiwan during pre-breeding migration which can explain the potential gene flow between Taiwan and mainland Japan populations although they might have different wintering sites.

In addition, the moderate to great differentiation revealed among the geographical groups (i.e., Taiwan, Okinawa and mainland Japan) may primarily link to the geographical distance between breeding colonies. Significant positive isolation by distance pattern was revealed from the analysis of this dataset and this indicates that there were connections but the geographical distances restrict the gene flow among the populations. The gene flow among the populations decreased when the geographic distance increased causing the larger genetic difference between the populations with larger geographic distance (Wright 1943). The Little Terns utilize the coastal areas for breeding and foraging. During breeding period, they often forage within 4km from their colonies (Bertolero et al. 2005) and their movements are restricted since they have to frequently feed their chicks. Therefore, three geographical groups in this study are probably isolated by the open ocean, as suggested for other coastal seabird species in previous studies (Friesen 2015). Although seabirds including the Little Tern exhibit high dispersal abilities, but the breeding birds can usually disperse to increase their

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reproductive success (Steiner and Gaston 2005). Seabirds prefer to disperse to a nearby colony for breeding rather than spending high energy cost for long-distance dispersal (Antaky et al. 2021). So far, banding and resight data do not reveal that the Taiwanese Little Terns breed in the areas outside of Taiwan, for example, in Japan. This may indicate that the Taiwanese Little Terns come back to Taiwan and may only disperse to the areas in and around Taiwan. They do not simply spend energy on dispersing to a distant site for breeding, such as to Okinawa or mainland Japan by crossing the open oceans. The Little Terns in Taiwan may not return to the exact or nearby site for breeding but they may breed at the other sites located in Taiwan. This may also be true for the Little Tern populations in Okinawa and mainland Japan. Furthermore, seabirds that breed in the same environment experienced during their early life benefit them to select their groups and adapt to the local environments (Mancilla-Morales et al. 2022).

4.4 Conservation implications

Seabirds utilize large scale of habitat and can quickly response to the changing environmental conditions, so they are good indicators for the health of marine ecosystems and they may show the status of habitat, declination in food occurrence and abundance and rate of the predation in the inhabited areas (Rajpar et al. 2018). For instance, monitoring the Little Tern populations can help us to monitor the stock of their preys (i.e., the anchovy stocks) surrounded the waters of Taiwan. Previously, the monitoring data of the Little Tern populations in Taiwan were collected annually by regional bird societies with respect to the regions and/or counties of the breeding sites located. The Little Tern populations in Taiwan were concluded to be in declining trend based on the data collected from the main Little Tern colonies (i.e., colonies in Penghu, Zhanghua and Yilan) in recent years (NTU Biodiversity Center 2020). On the other hand, newly established

doi:10.6342/NTU202300368

colonies are continuing to be discovered over time, such as in Taoyuan, Hualien (NTU Biodiversity Center 2020) and Tainan (Wang 2022). This indicates the decreasing Little Tern populations were probably not due to failure on reproduction and survival, instead they disperse from the old sites to new sites to mitigate the impacts on reproductive success. Since the present study showed no structure and suggested high inter-colony dispersal among the populations, so all colonies of the Little Terns in Taiwan should be considered as a single panmictic population. All of the breeding sites of the Little Tern in Taiwan should be managed as a single protection unit when planning the conservation strategies. Thus, the population data collected from each region are suggested to be unified according to collection years to give a clear picture of the annual range for the Little Tern populations in Taiwan. In that case, the fluctuation on the Little Tern populations can be monitored effectively to know whether the decreasing of the colonies is associated with increasing of other colonies. Accordingly, we can determine whether the fluctuation was caused by reproductive success or failure and/or inter-colony dispersal and adjust the conservation strategies in response to changing conditions.

High genetic variation and connectivity among populations found in this study means that the destruction of a local population may not cause a severe negative effect on the genetic diversity of the Little Tern populations as whole in Taiwan if the other suitable habitats are readily available to accommodate the colonies emigrated from the destructed habitats. However, the genetic variation may not exactly reflect the current processes since the populations are showed not to be in mutation-drift equilibrium. This study points out some potential risks in eastern side of the Little Tern populations (i.e., PH and ZH) which may bring negative effects and may only reflect on genetic diversity when the populations reached the equilibrium. If the potential risks do bring negative effects to the populations, the current high genetic diversity detected means it is still not too late to

doi:10.6342/NTU202300368

enhance our conservation actions. High connectivity among breeding colonies may help the populations to buffer against genetic loss but long-term threats that cause the population decline may cancel out the buffering effects (Ramírez et al. 2013). From this, we should maintain the quality of the Little Tern habitats to retain maximally habitat availability in different regions, in order to maintain the current genetic diversity and connectivity among the populations across regions in Taiwan. We should set a higher priority on conserving the populations that faced potential risks, especially the western sides populations. We should prevent stray dogs from approaching the breeding sites of Little Terns in PH by restricting the release of stray dogs around the breeding sites. The development planning actions that affect the Little Tern habitat would need to be considered in a spatial and temporal context because fewer and smaller habitat range are available to birds through time.

Additionally, we could determine the philopatry, site fidelity and dispersal ability of the Little Tern to understand the extent of connectivity among the colonies between or within western and eastern sides of Taiwan when planning management actions, like how frequent the colonies connected to each other and the factors that promote the connectivity. Increasing sampling size and extending range of sampling areas across all breeding colonies in Taiwan are needed for the future work to provide a more comprehensive understanding of the connectivity among the colonies and their population demography. Geolocator studies that gather information on the migratory routes and wintering grounds of the Little Tern populations of different origins should also be conducted to clearly understand the factors in constraining or retaining gene flow among the populations in Taiwan as well as in Japan.

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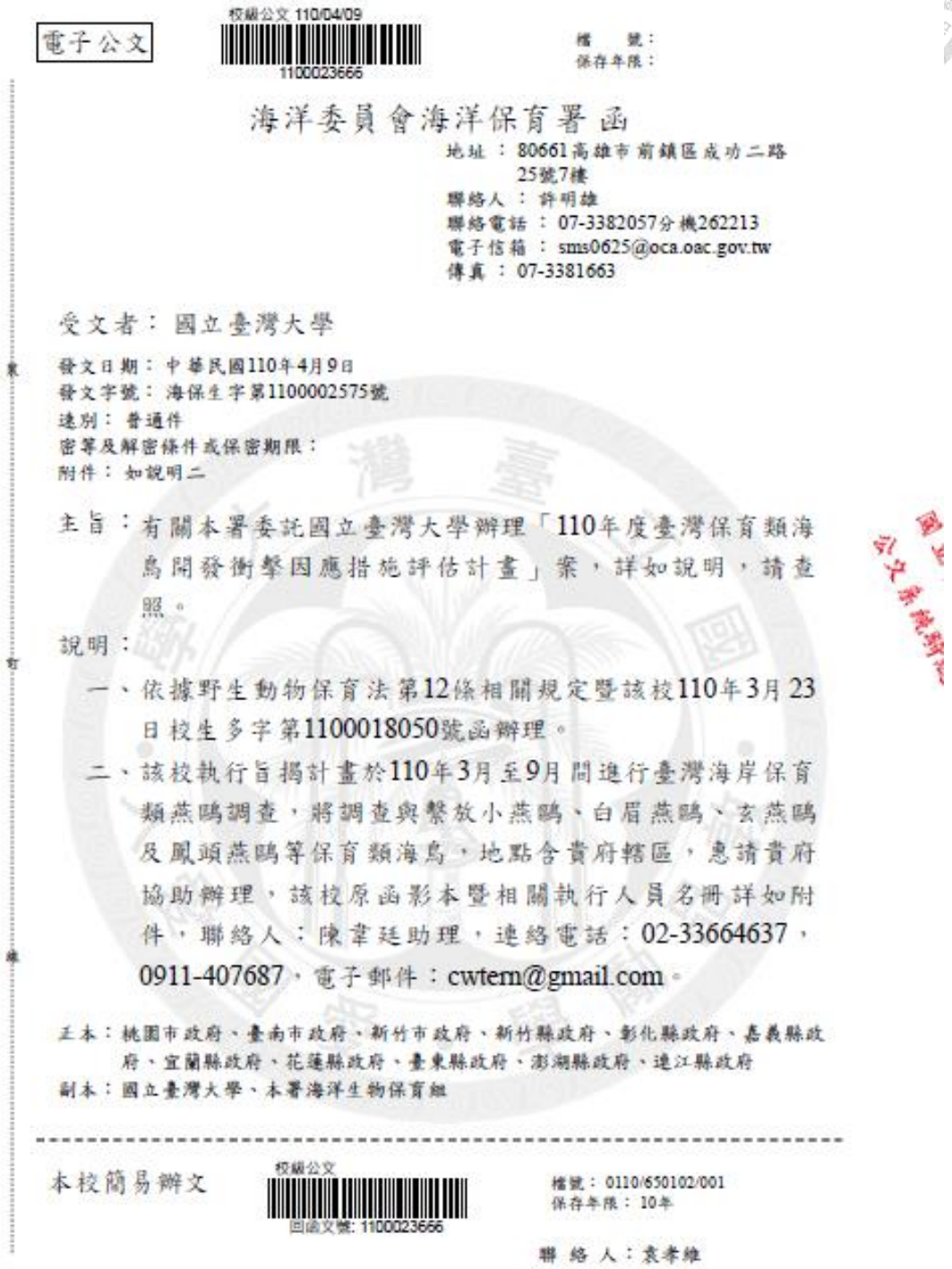
Appendix I. The scanned documents of sampling permission for the Little Tern in Taiwan in year 2021.



電子公文

校級公文 110/04/09
1100023666

檔　號：
保存年限：

海洋委員會海洋保育署 函

地址：80661高雄市前鎮區成功二路25號7樓
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受文者：國立臺灣大學

發文日期：中華民國110年4月9日
發文字號：海保生字第1100002575號
速別：普通件
密等及解密條件或保密期限：
附件：如說明二

主旨：有關本署委託國立臺灣大學辦理「110年度臺灣保育類海鳥開發衝擊因應措施評估計畫」案，詳如說明，請查照。

說明：

一、依據野生動物保育法第12條相關規定暨該校110年3月23日校生多字第1100018050號函辦理。

二、該校執行旨揭計畫於110年3月至9月間進行臺灣海岸保育類燕鷗調查，將調查與繫放小燕鷗、白眉燕鷗、玄燕鷗及鳳頭燕鷗等保育類海鳥，地點含貴府轄區，惠請貴府協助辦理，該校原函影本暨相關執行人員名冊詳如附件，聯絡人：陳韋廷助理，連絡電話：02-33664637，0911-407687，電子郵件：cwtern@gmail.com。

正本：桃園市政府、臺南市政府、新竹市政府、新竹縣政府、彰化縣政府、嘉義縣政府、宜蘭縣政府、花蓮縣政府、臺東縣政府、澎湖縣政府、連江縣政府
副本：國立臺灣大學、本署海洋生物保育組

本校簡易辦文

校級公文
回函文號: 1100023666

檔號：0110/650102/001
保存年限：10年

聯絡人：袁孝維

第1頁，共2頁

doi:10.6342/NTU202300368

Appendix II. The scanned documents of sampling permission for the Little Tern in Taiwan in year 2022.

抄本

檔　　號：
保存年限：

海洋委員會海洋保育署　函

機關地址：80661高雄市前鎮區成功二路25號7樓
聯 絡 人：許明雄
聯絡電話：07-3382057 #262213
傳真電話：07-3381663
電子郵件：sms0625@oca.oac.gov.tw

受文者：
發文日期：中華民國111年5月17日
發文字號：海保生字第1110004637號
速別：普通件
密等及解密條件或保密期限：
附件：如說明

主旨：有關本署委託國立臺灣大學辦理「111年度海鳥族群調查」案，詳如說明，請查照。

說明：

一、依據野生動物保育法第12條相關規定暨國立臺灣大學111年5月9日校生多字第1110030213號函辦理。

二、該校執行旨揭計畫將搜尋及繫放小燕鷗保育類海鳥，地點含貴府轄區，該校原函影本暨執行人員名冊等相關資料詳如附件；該校聯絡人：洪崇航博士，連絡電話：0919-568081，電子郵件：chrancor@gmail.com。

正本：桃園市政府、新竹市政府、新竹縣政府、嘉義縣政府、彰化縣政府、宜蘭縣政府、花蓮縣政府、澎湖縣政府
副本：本署海洋生物保育組

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Appendix III. The scanned documents of affidavit of approval of animal use protocol for the Little Tern in Taiwan in year 2021.

國立臺灣大學動物實驗申請案審查同意書

Affidavit of Approval of Animal Use Protocol

National Taiwan University

動物實驗申請表暨同意書編號： 110 實證字第 00036 號

計畫申請人：袁孝維　　職　稱：教授

單位：國立台灣大學森林環境暨資源學系

飼養/應用地點：本實驗未進行任何動物飼養及管理，所有動物皆從野外捕捉，進行量測與紀錄後現場野放，並未攜回實驗室或籠舍。

計畫名稱：110 年度臺灣保育類海鳥開發衝擊因應措施評估計畫

本計畫之「動物實驗申請表」業經實驗動物照護及使用委員會

■ 實質 □ 形式審查通過。

本計畫預定飼養應用之動物如下：

動物種類	動物數量/年度	計畫執行期間
其它/小燕鷗/*Sternula albifrons*	10/110	110 年 06 月 28 日至 110 年 12 月 31 日

The animal use protocol listed below has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).
Protocol Title: Impact assessment for endangered seabird in Taiwan
IACUC Approval No: NTU-110-EL-00036
Period of Protocol: Valid From: 2021/06/28 To: 2021/12/31
Principal Investigator (PI): Hsiao-Wei Yuan

動物實驗計畫書審核小組召集委員：劉逸軒

實驗動物照護及使用委員會召集委員：　　日期：110 年 06 月 28 日

IACUC Chairman ***I-Hsuan Liu, DVM, PhD***　　Date 2021/06/28

Institutional Animal Care and Use Committee (IACUC) NTU

doi:10.6342/NTU202300368

Appendix IV. The scanned documents of affidavit of approval of animal use protocol for the Little Tern in Taiwan in year 2022.

國立臺灣大學動物實驗申請案審查同意書

Affidavit of Approval of Animal Use Protocol

National Taiwan University

動物實驗申請表暨同意書編號： 111 實證字第 00047 號

計畫申請人：袁孝維　　職 稱：教授

單位：國立臺灣大學森林環境暨資源學系

飼養/應用地點：野外捕捉，無須飼養於動物舍中

計畫名稱：111 年度海鳥族群調查

本計畫之「動物實驗申請表」業經實驗動物照護及使用委員會

■ 實質 □ 形式審查通過。

本計畫預定飼養應用之動物如下：

動物種類	動物數量/年度	計畫執行期間
其它/小燕鷗/*Sternula albifrons*	10/111	111 年 05 月 31 日至 111 年 12 月 20 日

The animal use protocol listed below has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).
Protocol Title: 2022 Seabird population survey
IACUC Approval No: NTU-111-EL-00047
Period of Protocol: Valid From: 2022/05/31 To: 2022/12/20
Principal Investigator (PI): Hsiao-Wei Yuan

動物實驗計畫書審核小組召集委員：
實驗動物照護及使用委員會召集委員：　劉逸軒　　日期：111 年 05 月 31 日

IACUC Chairman ***I-Hsuan Liu, DVM, PhD***　　Date 2022/05/31

Institutional Animal Care and Use Committee (IACUC) NTU

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Appendix V. The supplementary materials in this study.

Table S1. Classification and geographic locality of the species in genus *Sternula* and their Genbank accession numbers for *COI* gene analyses.

Genus	Species	Locality	Individual ID	Accession No.
Sternula	*S. albifrons*	Australia	EU525516	EU525516
			EU525518	EU525518
			EU525510	EU525510
			EU525522	EU525522
			MK262668	MK262668
		East Africa	MF580153	MF580153
			MF580179	MF580179
			MF580180	MF580180
			MF580182	MF580182
			MF580197	MF580197
			MF580207	MF580207
		Korea	NC028176	NC028176
			KT350612	KT350612
		Iraq	JF498813	JF498813
		Japan	AB843175	AB843175
			AB843176	AB843176
			AB843177	AB843177
			AB843771	AB843771
			AB843772	AB843772
			AB843773	AB843773
			JF499160	JF499160
			JF499161	JF499161
			JF499162	JF499162
			JF499163	JF499163
		Netherlands	KF946861	KF946861
		Russia	GQ482670	GQ482670
			GQ482671	GQ482671
		Sweden	GU571631	GU571631
			GU572100	GU572100
			GU572101	GU572101
		Taiwan	QL003	This study

doi:10.6342/NTU202300368

QL004	This study
QL007	This study
QL009	This study
QL010	This study
QL016	This study
QL017	This study
QL018	This study
QL022	This study
QL025	This study
QL060	This study
QL061	This study
QL063	This study
QL064	This study
ZB027	This study
ZB028	This study
ZB030	This study
ZB031	This study
ZB034	This study
ZB037	This study
ZB039	This study
ZB040	This study
ZB042	This study
NA043	This study
NA044	This study
NA045	This study
NA049	This study
NA051	This study
NA053	This study
NA054	This study
NA055	This study
NA056	This study
NA057	This study
NA058	This study
NA075	This study
NA076	This study
NA077	This study

doi:10.6342/NTU202300368

		NA078	This study
		HL082	This study
		HL083	This study
		HL084	This study
		HL085	This study
		HL086	This study
		HL087	This study
		HL088	This study
		HL089	This study
		HL090	This study
	United Kingdom	EU525517	EU525517
		EU525519	EU525519
		EU525521	EU525521
S. antillarum	USA, California	DQ433206	DQ433206
		JQ176284	JQ176284
		JQ176285	JQ176285
	USA, Florida	DQ433205	DQ433205
		KJ013276	KJ013276
	USA, Louisiana	EU525523	EU525523
		EU525524	EU525524
S. nereis	New Zealand	MK261972	MK261972
		MK262011	MK262011
		MK262098	MK262098
		MK262186	MK262186
		MK262624	MK262624
S. superciliaris	Brazil	EU525525	EU525525
		EU525526	EU525526
		EU525527	EU525527
		FJ028327	FJ028327
		FJ028328	FJ028328

doi:10.6342/NTU202300368

Table S2. Parameter settings for different SNP sets [#14: 0.85].

Min samples per locus for output [21]	SNPs	Missing data (%)
25	12263	49.57
26	11087	48.53
27	10028	47.71
28	8968	46.85
29	7732	45.68
30	6657	44.57
31	5733	43.62
32	4711	42.91
33	3710	41.55
34	3111	40.59
35	2543	39.18

Table S3. Number of loci recovered for the different datasets under different ipyrad clustering parameters [#21: 31].

Clustering parameter [14]	SNPs	Missing data (%)
0.85	5733	43.62
0.90	6133	42.93
0.95	5438	42.11

Table S4. Parameter settings for different SNP sets [#14: 0.90].

Min samples per locus for output [21]	SNPs	Missing data (%)
31	6133	42.93
32	5113	42.24
33	4080	41.03
34	3400	40.09

doi:10.6342/NTU202300368

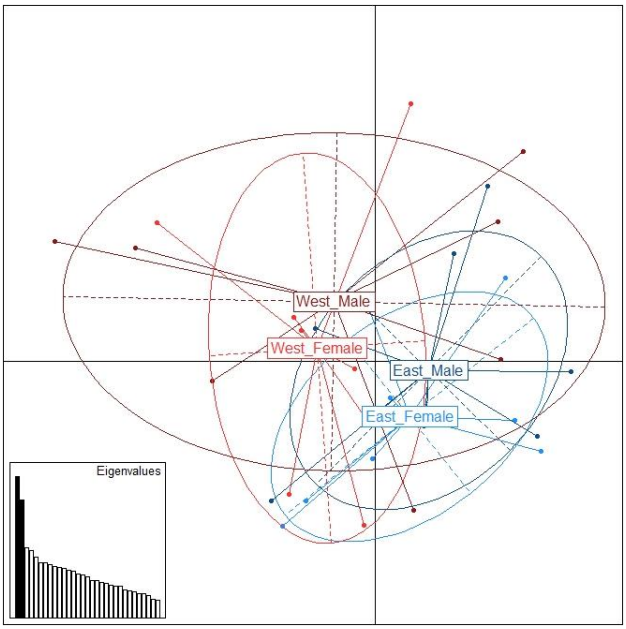

Figure S1. Principal Component Analysis (PCA) for the ddRAD sequencing data of Little Tern samples according to sex which collected from western (red) and eastern (blue) sides of Taiwan. The female individuals were represented by light colours while male individuals were represented by dark colours. The PC1 explained 9.91% while PC2 explained 8.27%.

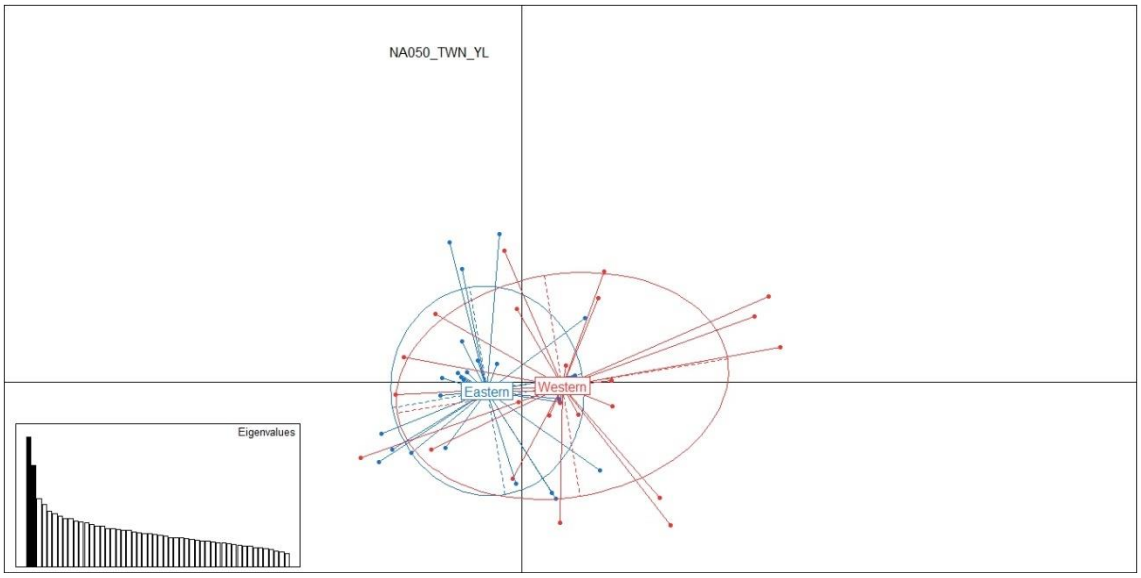

Figure S2. Principal Component Analysis (PCA) for the ddRAD sequencing data of the samples (n = 51) collected using the same parameter settings as in this study. The samples included the confirmed Little Tern samples from western (red) and eastern (blue) sides of Taiwan and the suspected Least Tern sample NA050_TWN_YL (black) collected in Yilan, Taiwan. The individuals in different groups were represented by different colours. The PC1 explained 7.17% while PC2 explained 5.59%.

doi:10.6342/NTU202300368

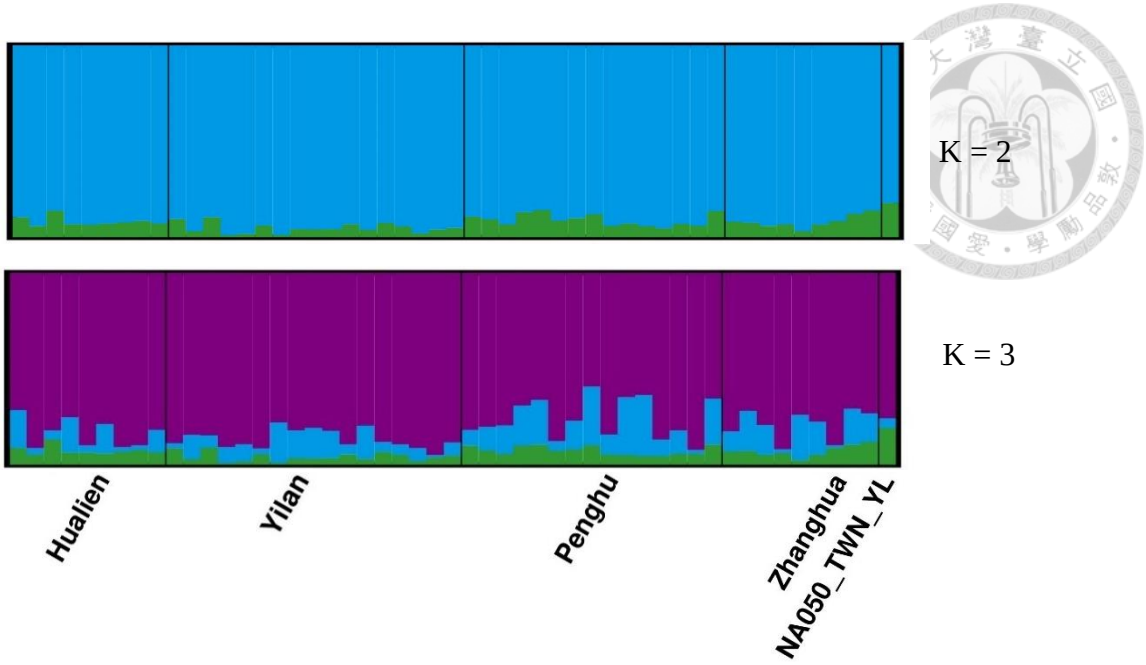

Figure S3. Results of clustering analysis for the ddRAD sequencing data of the samples (n = 51) collected using the same parameter settings as in this study. The samples included the confirmed Little Tern samples and the suspected Least Tern sample (NA050_TWN_YL) inferred by STRUCTURE for K = 2-3 (10 iterations per K). The Little Tern samples are grouped by sampling sites.

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