ความหลากหลายทางพันธุกรรมของลิงหางยาว *Macaca fascicularis* และ
ลิงวอก *M. mulatta*: เน้นศึกษาบริเวณที่มีการผสมข้ามสายพันธุ์

นางสาวศรีจันทร์ บัลลังทรัพย์

จุฬาลงกรณ์มหาวิทยาลัย
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บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
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GENETIC DIVERSITY OF LONG-TAILED MACAQUE *Macaca fascicularis* AND RHESUS MACAQUE *M. mulatta*: MAINLY FOCUS ON THEIR HYBRIDIZATION RANGE

Miss Srichan Bunlungsup

จุฬาลงกรณ์มหาวิทยาลัย
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A Dissertation Submitted in Partial Fulfillment of the Requirements
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Thesis Title	GENETIC DIVERSITY OF LONG- TAILED MACAQUE *Macaca fascicularis* AND RHESUS MACAQUE *M. mulatta*: MAINLY FOCUS ON THEIR HYBRIDIZATION RANGE
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ศรีจันทร์ บัลลังทรัพย์ : ความหลากหลายทางพันธุกรรมของลิงหางยาว *Macaca fascicularis* และลิงวอก *M. mulatta*: เน้นศึกษาบริเวณที่มีการผสมข้ามสายพันธุ์ (GENETIC DIVERSITY OF LONG-TAILED MACAQUE *Macaca fascicularis* AND RHESUS MACAQUE *M. mulatta*: MAINLY FOCUS ON THEIR HYBRIDIZATION RANGE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. สุจินดา มาลัยวิจิตรนนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. David Glenn Smith, รศ. ดร. Sreetharan Kanthaswamy, 169 หน้า.

ลิงหางยาวชนิดย่อยปกติ (*Macaca fascicularis fascicularis, Mff*) เป็นหนึ่งในสัตว์ไพรเมทที่นิยมนำมาใช้เป็นสัตว์ทดลองในงานวิจัยทางด้านชีวการแพทย์ ดังนั้นลักษณะทางพันธุกรรมของ *Mff* จึงเป็นที่สนใจกันมาก แต่ด้วย *Mff* มีอาณาเขตการแพร่กระจายที่ใกล้ชิดกันมากกับลิงวอก (*M. mulatta, Mm*) ที่บริเวณ 15-20 องศาเหนือ และกับลิงหางยาวชนิดย่อยพม่า (*M. fascicularis aurea, Mfa*) ที่บริเวณ 8-12 องศาเหนือในประเทศไทยและเกิดการผสมข้ามสายพันธุ์ขึ้น งานวิจัยนี้จึงศึกษาการผสมข้ามสายพันธุ์ทั้งในระดับชนิด (*Mm* x *Mff*) และระดับชนิดย่อย (*Mff* x *Mfa*) ของ *Mff* ที่มีถิ่นกำเนิดในประเทศไทยและบริเวณใกล้เคียง โดยวิเคราะห์ไมโตคอนเดรียลดีเอ็นเอ (mtDNA) วายโครโมโซม (Y-chromosome) และสนิปส์ (SNPs) จากการวิเคราะห์วงศ์วานวิวัฒนาการด้วย mtDNA และ Y-chromosome พบว่าการผสมข้ามสายพันธุ์ระหว่าง *Mm* และ *Mff* เกิดในทิศทางเดียว คือ จาก *Mm* เพศผู้รุกรานเข้าไปในฝูง *Mff* โดยมีจุดสิ้นสุดอยู่ใต้คอคอดกระระหว่าง SSD (จังหวัดชุมพร) และ WSK (จังหวัดพังงา) แต่จากการวิเคราะห์ SNPs พบว่าการผสมข้ามสายพันธุ์ระหว่าง *Mm* และ *Mff* สามารถเกิดขึ้นได้ทั้ง 2 ทิศทาง ทั้งจาก *Mm* สู่ *Mff* และในทางกลับกัน เพียงแต่การถ่ายทอดพันธุกรรมของ *Mm* เข้าสู่ฝูง *Mff* มีระยะทางมากกว่า (ประมาณ 10 องศาละติจูด) จาก *Mff* ถ่ายทอดพันธุกรรมสู่ฝูง *Mm* (ประมาณ 4 องศาละติจูด) โดยคาดว่าการถ่ายทอดพันธุกรรมของ *Mm* เข้าไปในฝูง *Mff* มีจุดสิ้นสุดอยู่ระหว่าง KNKTK (จังหวัดสงขลา) และ สิงคโปร์/ซาราวัค อาศัยผลการวิเคราะห์ mtDNA ร่วมกับการเปลี่ยนแปลงทางภูมิศาสตร์ในอดีตงานวิจัยนี้ยังได้เสนอสมมติฐานการเกิดวิวัฒนาการของ *Mff* ที่มีการอพยพข้ามผืนแผ่นดินที่เชื่อมต่อระหว่างเขตอินโดจีนและซุนดาในช่วงยุคน้ำแข็ง (glacial) และระหว่างยุคน้ำแข็ง (interglacial) อีกด้วย จากการวิเคราะห์วงศ์วานวิวัฒนาการด้วย mtDNA และ Y-chromosome สามารถแบ่งลิงลูกผสมระหว่าง *Mff* และ *Mfa* ออกเป็น 2 กลุ่ม คือ กลุ่มอินโดจีน (SRY, KRI, WKC, and BMS) และกลุ่มซุนดา (KRI) โดยคาดว่าการผสมข้ามสายพันธุ์ระดับชนิดย่อยมี 2 รูปแบบ คือ *Mfa* เพศผู้เคลื่อนจากแผ่นดินใหญ่ของเมียนมาร์ลงมาทางใต้ ผ่านหมู่เกาะมะริดเข้าสู่ชายฝั่งอันดามัน รุกรานเข้าสู่ฝูง *Mff* และเกิดเป็นลิงลูกผสมกลุ่มซุนดาขึ้น ในขณะที่ลิง *Mfa* เพศผู้บางตัวเมื่อเคลื่อนมาถึงหมู่เกาะมะริด ได้อพยพต่อไปทางทิศตะวันออกเฉียงเหนือข้ามเทือกเขาตะนาวศรีบริเวณที่ไม่สูงนักไปทางฝั่งอ่าวไทย รุกรานเข้าสู่ฝูง *Mff* และเกิดเป็นลิงลูกผสมกลุ่มอินโดจีนขึ้น ทั้งนี้จากการวิเคราะห์วงศ์วานวิวัฒนาการของ *Mfa* ด้วย mtDNA ที่พบ *Mfa* แยกออกมาจาก *Mff-Mm* และด้วย *Mfa* เป็นลิงมะแคคเพียงชนิดเดียวจากทั้งหมด 22 ชนิด ที่สามารถใช้เครื่องมือหินในการหาอาหารได้ จึงทำให้เกิดคำถามเกี่ยวกับสถานะทางอนุกรมวิธานของ *Mfa* ที่มีอยู่ในปัจจุบัน โดยสรุป การศึกษานี้ชี้ให้เห็นถึงความซับซ้อนทางพันธุกรรมของ *Mff* ที่เกิดจากกระบวนการทางวิวัฒนาการและการผสมข้ามสายพันธุ์กับทั้ง *Mm* และ *Mfa* ดังนั้นในการคัดเลือก *Mff* มาใช้ในงานวิจัยทางด้านชีวการแพทย์จึงต้องคำนึงถึงทั้งชนิดหรือชนิดย่อยของสัตว์และแหล่งที่มาไปพร้อมกัน

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KEYWORDS: HYBRIDIZATION / MACACA FASCICULARIS FASCICULARIS / MACACA FASCICULARIS AUREA / ISTHMUS OF KRA / MTDNA

SRICHAN BUNLUNGSUP: GENETIC DIVERSITY OF LONG-TAILED MACAQUE *Macaca fascicularis* AND RHESUS MACAQUE *M. mulatta*: MAINLY FOCUS ON THEIR HYBRIDIZATION RANGE. ADVISOR: PROF. SUCHINDA MALAIVIJITNOND, Ph.D., CO-ADVISOR: PROF. DAVID GLENN SMITH, Ph.D., ASSOC. PROF. SREETHARAN KANTHASWAMY, Ph.D., 169 pp.

Macaca fascicularis fascicularis (*Mff*) are one of the most popularly used non-human primate models in biomedical research and thus their genetic characteristics are a focal point of interest. However, *Mff* live parapatrically with *M. mulatta* (*Mm*) at 15-20° N and with *M. fascicularis aurea* (*Mfa*) at 8-12° N in Thailand and became hybridized. Using mtDNA, Y-chromosome and SNPs genetic markers, this study aims to determine the hybridization at interspecific (*Mff* x *Mm*) and intersubspecific (*Mff* x *Mfa*) levels of the *Mff* originated from Thailand and vicinity. Based on the mtDNA and Y-chromosome phylogenies, it indicated that hybridization between *Mm* and *Mff* was driven uni-directionally by the introgression of *Mm* males into *Mff* populations. The *Mm* gene flow directed southward and was restricted south of the Isthmus of Kra between SSD (Chumphon) and WSK (Phang-nga). In contrast, SNP genotyping revealed a bi-directional hybridization where genetic introgression was either from *Mm* into *Mff* or vice versa, however the gene flow from *Mm* into *Mff* was far greater (~10° latitude of distant) than from *Mff* into *Mm* (~4° latitude). This caused the *Mm* gene flow far beyond the Isthmus of Kra which proposed to terminate somewhere between the KN/KTK (Songkla) and Singapore/Sarawak. The migration scenario of *Mff* across land bridge during the glacial and interglacial cycles was also proposed based on the mtDNA phylogeny as well as changes of zoogeographical barriers. Based on the mtDNA and Y-chromosome phylogenies, the *Mff* x *Mfa* hybrids were subdivided into two groups; Indochinese (SRY, KRI, WKC, and BMS) and Sundaic (SRI) hybrids. Two hybridization scenarios were proposed. One is that *Mfa* males migrated from mainland Myanmar southward along Mergui archipelago toward the Andaman sea coast, introgressed into *Mff* populations and formed the Sundaic *Mff* x *Mfa* hybrids. Another is that some *Mfa* males migrated northeastward across the low altitude area of the Dawna range to the Thai Gulf, while they were along Mergui Archipelago, introgressed into *Mff* populations and formed the Indochinese *Mff* x *Mfa* hybrid. Since *Mfa* clade was separated from the *Mff-Mm* clade in mtDNA phylogenetic tree and it is the only one of 22 macaque species performing stone tool using behavior, the taxonomic status of this subspecies is now being questioned. In conclusion, this study unravels a complexity in *Mff* genomes based on their evolutionary history and hybridizations either with *Mm* or *Mfa*. Thus, the selection and use of *Mff* for biomedical research should consider not only species or subspecies identification, but also their origins.

Department: Biology
Field of Study: Zoology
Academic Year: 2016

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

BCG	Bacillus Calmette–Guérin
BDM	Ban Dong Muang
BIC	Bayesian information criterion
BMS	Banmaisomboon School
BNT	Bayin Nyi Temple
bp	Base pair
BRT	Barastagi
BSS	Bann Sang School
ca.	Circa
CI	Credibility Interval
CMC	Ca Mau Conservation
CRL	Crown rump length
EDTA	Ethylenediaminetetraacetic acid
EH	Expected heterozygosity
ESS	Estimated sample size
F_{IS}	Inbreeding coefficient
GPS	Global Positioning System
HVSI	Hyper Variable Segment I
IOK	Isthmus of Kra

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JLI	Jarlan Island (Lord Loughbrough)
Ka	Thousand year ago
KCS	Khao Chai Son
KN	Wat Khao Nor
KN/KTK	Khao Noi/Khao Tangkuan
KNG	Khao Ngu Rock Garden
KRI	Koram Islannd
KSP	Kosumphi Forest Park
KTM	Khao Tham Mee
KTP	Khao Toh Phyawang
LGM	last glacial period
LPI	Lampi Island
LTI	Lanta Island
MAF	Minor allele frequency
MCMC	Malkov Chain Monte Carlo
Mf	*Macaca fascicularis*
Mfa	*Macaca fascicularis aurea*
Mff	*Macaca fascicularis fascicularis*
MFRC	Mangrove Forest Research Center
ML	Maximum likelihood

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Mm	*Macaca mulatta*
Msyl	*Macaca sylvanus*
mtDNA	Mitochondrial DNA
MYA	Million year ago
NaCI	Sodium Chloride
NJ	Neighbor-joining
OH	Observed heterozygosity
PAR	Pseudoautosomal region
PNI	Panak Island
PNY	Piak Nam Yai Island
RTL	Relative tail length
SBG	Sibanganding
SDS	Sodium dodecyl sulfate
SIV	Simian immunodeficiency Virus
SKB	Sai Keaw Beach
SMT	Shin Ma Taung
SNPs	Single nucleotide polymorphisms
SRI	Sirae Island
SRY	Samroiyot National Park
SRY	Sex-determining region, Y-chromosome

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SSD	Suan Somdet Prasrinakharin, Chumphon
SSP	Suan Somdet Prasrinakharin, Phangnga
STRs	Short tandem repeats
SY	Sai Yok
TKH	Tham Khao Ha Yod
TKW	Tam Khao Keeree Wong
TL	Tail length
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TSPY	Testis-specific protein, Y-chromosome
WHM	Wat Haad Moon
WKC	Wat Khao Chong Krachok
WKCA	Wat Tham Khao Chaangon
WKH	Wat Khuha Phimuk
WKK	Wat Khao Keaw Wichian
WKS	Wat Khuha Sawan
WKT	Wat Khao Thamon
WPK	Wat Pikul Ngam
WPN	Wat Paknam Pracharangsarith
WPT	Wat Phattanajit
WSK	Wat Suwan Khuha

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WTM	Wat Thammasala
WTPMH	Wat Tham Pa Mak Ho
WTS	Wat Tham Sue
WTT	Wat Tham Thepbandan
ZDK	Zadetkyi
RTL	Relative tail length

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CHAPTER I
GENERAL INTRODUCTION

Old World monkeys in the genus *Macaca* or macaques comprise approximately 22 distinct species (Zinner et al., 2013). These species are categorized into 4 species groups; *silenus- sylvanus*, *fascicularis*, *sinica* and *arctoides*, based on the distinct morphologies of their glans penis (Fooden, 1976, 1980). Among these 22 macaque species, *Macaca mulatta* (rhesus macaque) and *M. fascicularis* (long-tailed macaque) in *fascicularis* species group have the largest and second largest geographic distribution among all non-human primate species (Wheatley, Stephenson, & Kurashina, 1999). *M. mulatta* range between *ca.* 15 to 36° N in the South, East and Southeast Asian continents, covering Afghanistan, Pakistan, Bangladesh, Bhutan, India, Nepal, China, Myanmar, Thailand, Laos and Vietnam. Their northern range is defined by the Great Indian desert, the Tibetan Plateau and the transition between mesothermal and microthermal climates (Fooden, 2000), and their southern range is restricted by the interspecific competition with *M. fascicularis*. *M. mulatta* are basically divided into three main groups of Chinese, Indian and Indochinese (Fooden, 2000; Hamada et al., 2006).

M. fascicularis are distributed throughout the mainland and islands of Southeast Asia between *ca.* 20° N to 10° S. They are found in Bangladesh, Myanmar, Thailand, Laos, Vietnam, Cambodia, Malaysia, Singapore, Brunei, Indonesia, the Philippines, Timor, Mauritius and Nicobar island which is a part of India (Fooden, 1995). Current classification claims that *M. fascicularis* comprises 10 different subspecies (Fooden, 1995, 2006; Groves, 2001). Among all of them, *M. fascicularis fascicularis* (common long-tailed macaque), *M. f. aurea* (Burmese long-tailed macaque), and *M. f. philippinensis* (Philippine long-tailed macaque) are the three major subspecies for research.

In the present days, both *M. mulatta* and *M. f. fascicularis* are popularly used as a non-human primate models in biomedical research. This is not only because they are

commercially available and commonly encounter, but their genetic characteristics are also closely related to those of humans. Compared to other animal models such as rats or mice, both these species of macaques are evolutionarily closer to humans. Macaques share a common ancestor with humans dating back approximately 25 million years ago (MYA) (Glazko & Nei, 2003) while the rodent-human split occurred approximately 60 MYA (Benton & Donoghue, 2007).

A major concern of using any animal models in biomedical research is the consistency of their genetic characteristics; because using animals with different genetic backgrounds may lead to irreproducible and therefore unreliable results. For example, *M. mulatta* are more susceptible to *Plasmodium knowlesi* and thus experience higher mortality rates than *M. fascicularis* (Schmidt et al., 1977). Since *M. mulatta* exhibit severe manifestation of disease after being infected and share more post-symptoms with the humans, they are more suitable for studying malaria (White, 2008). Thus, if a researcher uses *M. mulatta* with the genetic admixture with *M. fascicularis*, the results might not be as predictable as they would be influenced by the study subjects' varied genetic backgrounds rather than the procedures of an experimental trial. Moreover, in the case of macaques, wild-caught founders that are used in breeding programs within primate research centers throughout the world often have well-documented species identification, however information of their exact geographic origins may be lacking (Stevison & Kohn, 2008; Shiina et al., 2010). Genetic variation that reflects the differences in geographic origin among research subjects can also contribute to allele frequency heterogeneity and has implications for design of biomedical experiments. As such, macaques sourced from different origins could carry different genetic background and respond to the treatments in the different ways. For example, Rhodes et al. (2017) recommended that Indian *M. mulatta* and Indonesian *M. fascicularis* were the most suitable animal models to study the tuberculosis and translate the knowledge to the naive BCG-vaccinated humans, whereas Mauritian *M. fascicularis* are the best animal models for the translational research of tuberculosis in the BCG-vaccinated human.

Due to the geographic changes during glacial and inter-glacial periods, *M. mulatta* and *M. f. fascicularis* had been migrated southerly and northerly across their boundary and hybridized (Tosi, Morales, & Melnick, 2002). Their hybrid ranges have been proposed to be between 15 and 20° N, covering four countries including Myanmar, Thailand, Laos and Vietnam (Fooden, 1995, 2000; Hamada et al., 2006). Since the hybrid offspring of macaques are fertile, these interspecific hybrids can therefore pass their mixed genetic to the descendants (Bernstein & Gordon, 1980). The hybridization scenario including direction of the hybridization, expansion of the genetic admixture from the hybrid zone and terminal location of genetic introgression, have become a major focal point of interest for research over the past decade (Tosi et al., 2002; Kanthaswamy et al., 2008; Osada et al., 2010; Satkoski et al., 2013).

Other than the interspecific hybridization between *M. mulatta* and *M. f. fascicularis*, natural intraspecific hybridization between the two subspecies of *M. fascicularis*, *M. f. aurea and M. f. fascicularis*, has also been reported. The intraspecific contract zone has been recognized close to the Isthmus of Kra (Fooden, 1995) which locates between Ranong and Chumphon provinces, Thailand. Based on the locations where the hybridization between the two species of *M. mulatta* and *M. f. fascicularis* or interspecific hybridization, and between the two subspecies of *M. fascicularis*, *M. f. aurea and M. f. fascicularis*, or intraspecific hybridization, Thailand is likely an important study site for these phenomena. Thailand connects two different bioregions (Indochina and Sunda) and encompasses several biogeographical barriers such as Isthmus of Kra, Taninthanyi ranges and Surat thani-Krabi depression (Denduangboripant & Cronk, 2000; Hughes et al., 2011; Hughes, Round, & Woodruff, 2003; Malaivijitnond et al., 2012) which have an impact on animal distribution and hybridization.

Although many previous studies have investigated hybridization events between *M. mulatta* and *M. f. fascicularis*, those studies have mostly relied on either

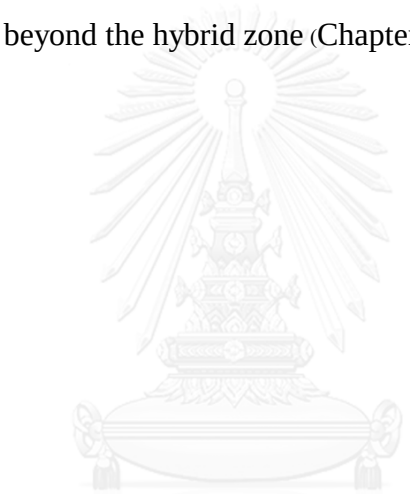
specimen from unknown geographic origins or samples that have the country of origin information due to the location of the primate breeding centers from which the samples were derived (Kanthaswamy et al., 2008, Bonhomme et al., 2009; Stevison & Kohn, 2009). The lack of animals with known provenances may have somewhat limited those studies concerning inter as well as intraspecific hybridization in the wild. On contrarily to the hybridization between *M. mulatta* and *M. f. fascicularis*, hybridization between *M. f. aurea and M. f. fascicularis* have not been reported prior to this thesis although morphological characteristics at the intersubspecific contact zone have been described (Fooden, 1995). Therefore, in this thesis, specimens of known origin *M. mulatta, M. f. fascicularis* and *M. f. aurea* throughout Thailand and its vicinity were collected to determine the interspecific and intraspecific hybridization between *M. mulatta* and *M. f. fascicularis* and between *M. f. aurea and M. f. fascicularis*, respectively.

As male macaques usually migrated out of the groups (male fission), and females stayed permanently in their natal group (female philopatry), mitochondria DNA (mtDNA) and Y-chromosome genes were selected to determine the process of the hybridization and direction of genetic introgression in this study. Autosomal single nucleotide polymorphisms (SNPs) and short tandem repeats (STRs) were analyzed to determine the degree of the genetic admixture at and beyond the interspecific hybrid zone between *M. mulatta* and *M. f. fascicularis* and at intraspecific zones between *M. f. fascicularis* and *M. f. aurea*, respectively.

It is anticipated that this study would shed light on the effects of inter and intraspecific hybridization on macaques' genetics which can be used as basic knowledge for the screening of *M. mulatta* and *M. f. fascicularis* in biomedical research.

Objectives

1. To determine the genetic diversity of *M. f. fascicularis* at their interspecific hybrid zones with *M. mulatta* and vicinity (Chapter III)
2. To evaluate the degree of genetic admixture between *M. f. fascicularis* and *M. mulatta* at and beyond the hybrid zone (Chapter IV)
3. To determine the genetic diversity of *M. f. fascicularis* at their intraspecific hybrid zones with *M. f. aurea* and vicinity (Chapter V)
4. To evaluate the degree of genetic admixture between *M. f. fascicularis* and *M. f. aurea* at and beyond the hybrid zone (Chapter VI)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CHAPTER II
LITERATURE REVIEW

1. The introduction to macaques

Non-human primates in the genus *Macaca* or macaque monkeys is one of the major lineages of the family Cercopithecidae (Old World monkey). They comprise over 20 species and have been subdivided into four to seven species group (Fooden, 1976; Fleagle 2013; Zinner et al. 2013). Fooden (1976) divided all macaques into four species groups of *silenus-sylvanus*, *fascicularis*, *sinica* and *arctoides*, based on their distinctive glans penis. Current classification based on genetic analysis suggests that there are seven species groups of *sylvanus*, *silenus*, *Sulawesi macaques*, *sinica*, *arctoides*, *mulatta* and *fascicularis* (Zinner et al., 2013). The latter classification spilts *M. sylvanus* (Barbary macaque) and all 6 species of Sulawesi macaques from *silenus-sylvanus* species group, and named them as *sylvanus* and the *Sulawesi macaque* species groups, respectively. *M. mulatta*, *M. fuscata* (Japanese macaque) and *M. cyclopis* (Taiwanese macaque) were separated from the *fascicularis* species group and named the *mulatta* species group.

1.1 *Macaca mulatta* (rhesus macaque)

Among these 22 species of macaques (Zinner et al., 2013), *M. mulatta* and *M. fascicularis* are the most and second most geographically distributed nonhuman primates throughout the Asian continent. *M. mulatta* are found in South, East, and Southeast Asia, *ca.* 15 to 36° N including Afghanistan, Pakistan, Bangladesh, Bhutan, India, Nepal, China, Myanmar, Thailand, Laos and Vietnam (Figure 2.1) (Fooden, 2000). Their common habitat types are broadleaf evergreen and mixed broadleaf-needleleaf forests, however they are occasionally found in needleleaf forests. Although most of their habitat altitudes are below 2,000 meters, they were also seen as high as 3,200 meters in Nepal and about 4,000 meters in the Qinghai province in China (Fooden,

2000). Their northern limits are determined primarily by physiographic and climatological factors; the Great Indian desert defined their north-western distribution, Himalayas and Xizang-Qinghai (Tibetan) Plateau restricted their north-central range while the transition zone between mesothermal and microthermal in China restricted their north-eastern range. Competition with neighboring species probably limit their south-eastern (competing with *M. fascicularis*) and south-western ranges (competing with *M. radiata*; bonnet macaque), respectively.

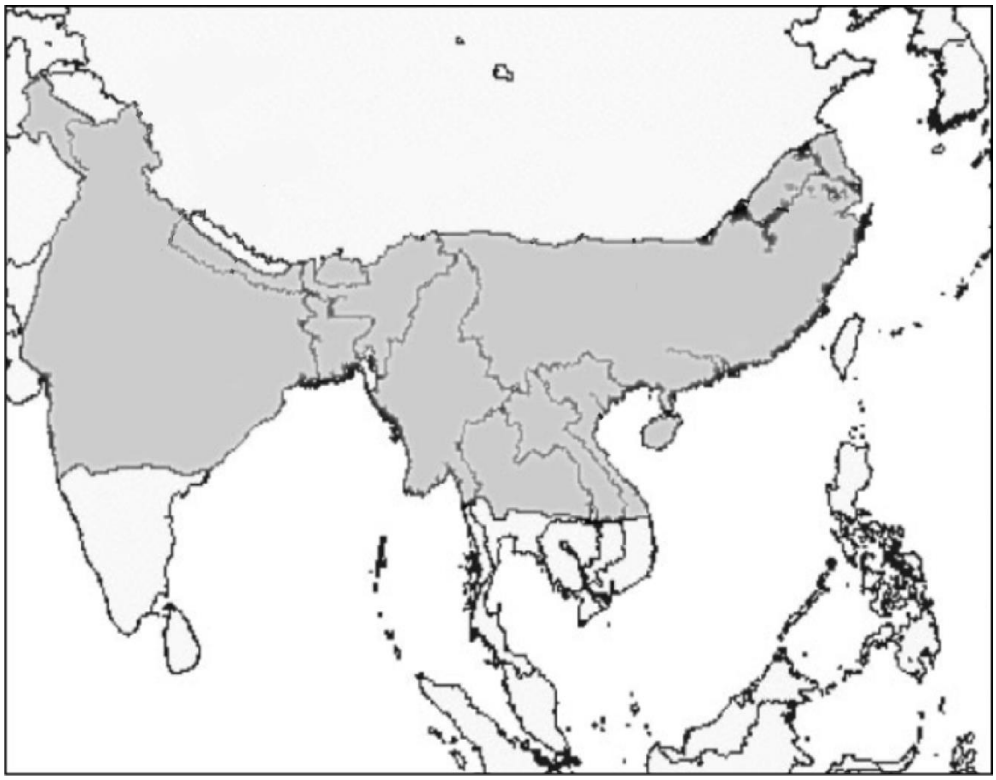

Figure 2. 1 The distribution range of *M. mulatta* is indicated by the gray color. (Adapted from Smith et al., 2005)

M. mulatta were previously classified into many subspecies (Hill, 1974, Jiang et al., 1991), however, due to inadequate information, the recognition of subspecies level

distinctions became obsolete and this species was subsequently divided into two main groups of Eastern (China and vicinity) and Western (India and vicinity) (Fooden, 2000). Hamada et al. (2006) later classified another rhesus group; Southern or Indochinese group which exhibit smaller body size and greater in their relative tail length than those of Chinese and Indian origins *M. mulatta* (Hamada et al., 2006).

Basically, there are two main morphological characters used for species identification in *M. mulatta*. The first character is their pelage color in which the lower part of their body is more yellowish or reddish and the upper part which varies from yellowish gray to golden brown to burnt orange (Fooden, 2000) (Figure 2.2). Another criterion is the ratio of tail length (TL) to crown rump length (CRL) resulting in the value of relative tail length (RTL). Comparisons among the three *M. mulatta* groups show that the Chinese *M. mulatta* possessed the lowest value of RTL (35%), followed by Indian (45%) and Indochinese (<70%) *M. mulatta*, respectively (Hamada et al., 2005, Hamada, San, & Malaivijitnond, 2016) (Figure 2.2). In addition to those two criteria, *M. mulatta* generally has whorl hair on their cheek or sometime forms an infrazygomatic pattern where the hairs of the temporal region are smoothly directed posteriorly from the posterior margin of the eye to the anterior margin of the ear (Fooden, 2000; Hamada et al., 2016) (Figure 2.2).

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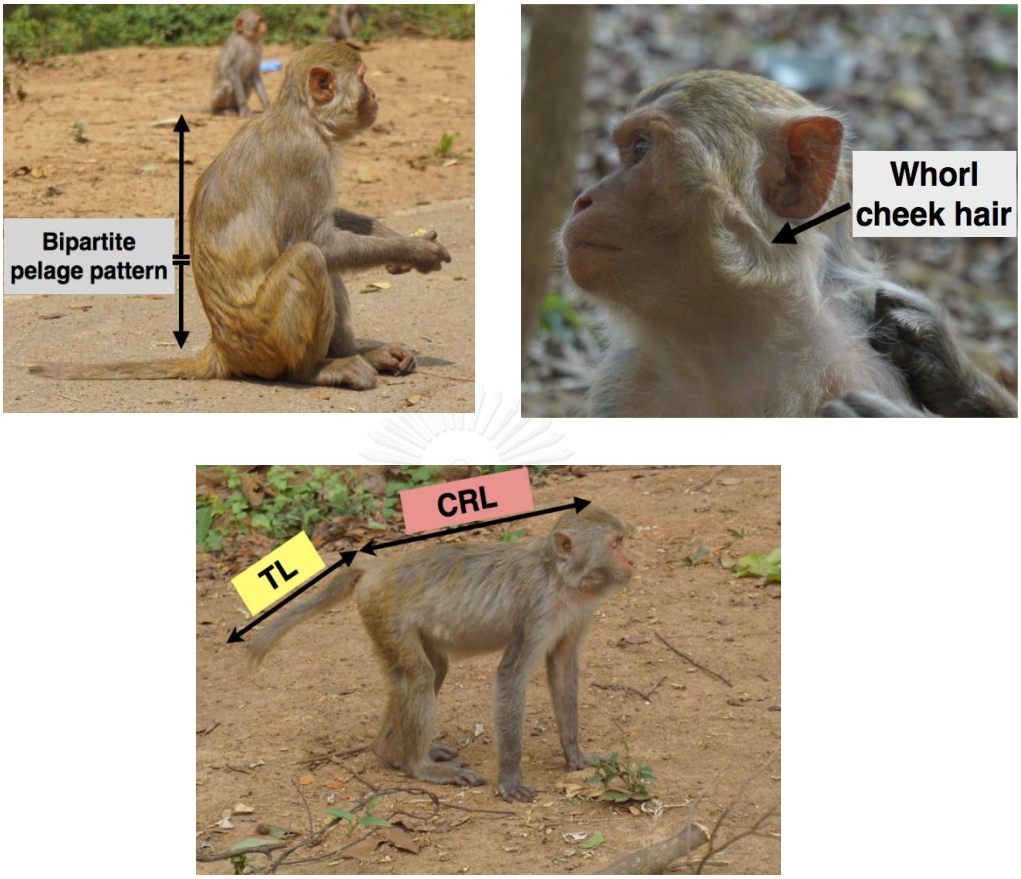

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Figure 2. 2 Morphological characteristics of *M. mulatta* (Indochinese group). TL and CRL stand for tail length and crown rump length, respectively.

1.2 *Macaca fascicularis* (long-tailed macaque)

M. fascicularis occupy wide habitat ranges that spread over mainland and insular Southeast Asia between *ca.* 20° N and 10° S (Fooden, 1997). This macaque is commonly encountered at a low elevation in the core area because no record above 400 meters in Vietnam, Cambodia, and Thailand has been found. However, they may extend to high altitude in some areas, for example; 1200 meters in west Malaysia or 2000 meters in Java (Fooden, 1995) . Their natural habitats include seashore, mangrove forest, riverbanks, swamp forest, primary and secondary forest in the Philippines, Indonesia, Malaysia, Singapore, Cambodia, Myanmar, Thailand, Laos and Vietnam (Fooden, 1995) . Since *M. fascicularis* can adapt very well to many habitat types, they are successful in colonizing many areas beyond their natural range including Mauritius, Island of Palau, West Papua, Tinjil Island near Java, Kabaena Island off the Sulawesi and Kowloon Hills of Hong Kong after introduction by humans (Gumert, 2011). Their natural northern range is defined by the interspecific competition with *M. mulatta* while the southern area was restricted by the deep-water.

Current classification divides *M. fascicularis* into 10 different subspecies; *M. fascicularis fascicularis*, *M. f. aurea*, *M. f. philippinensis*, *M. f. umbrosa*, *M. f. fusca*, *M. f. lasiae*, *M. f. atriceps*, *M. f. condorensis*, *M. f. tua* and *M. f. karimondjawe*, based on their distribution range and morphological characteristics (Fooden, 1995). Among these 10 subspecies, *M. f. fascicularis* or common long-tailed macaques have spread the widest and are found on the core area throughout mainland Southeast Asia, Java, Sumatra, Borneo and many of those shallow and deep-water fringing islands. Two other major subspecies are *M. f. aurea* and *M. f. philippinensis*. *M. f. aurea* or Burmese long-tailed macaques are found along the western range of the Indochinese *M. f. fascicularis*, i.e., from Bangladesh throughout Myanmar and its Mergui Archipelago, and southward near to the Isthmus of Kra in Thailand. Recently, *M. f. aurea* have been reported as possibly extinct in Bangladesh (Kabir &Ahsan 2012). Interestingly, *M. f. aurea* possess a prominent behavioral character not observed in other subspecies of *M. fascicularis*, they

have been reported to use stone tools to access encased foods such as nut, shellfish and oyster and as such are known as "tool-using macaques" (Gumert, Kluck, & Malaivijitnond, 2009; Gumert, Hoong, & Malaivijitnond, 2011; Gumert, Hamada, & Malaivijitnond, 2013; Gumert & Malaivijitnond, 2012, 2013; Malaivijitnond et al., 2007). The last main subspecies of *M. fascicularis* is *M. f. philippinensis*. This subspecies spread throughout the Philippines except in the south where *M. f. fascicularis* inhabit (Smith et al., 2014). The other remaining seven subspecies of *M. fascicularis* live on small isolated islands (Figure 2.3).

M. fascicularis do not have any bipartite patterns of pelage color as seen in *M. mulatta* (Figure 2.4). Their pelage color varies from buffy to yellowish gray to golden brown to reddish brown to blackish. They have infrazygomatic or transzygomatic check hair pattern together with a very long tail of more than 90% of RTL (Fooden, 1995; Hamada et al., 2016) (Figure 2.4).

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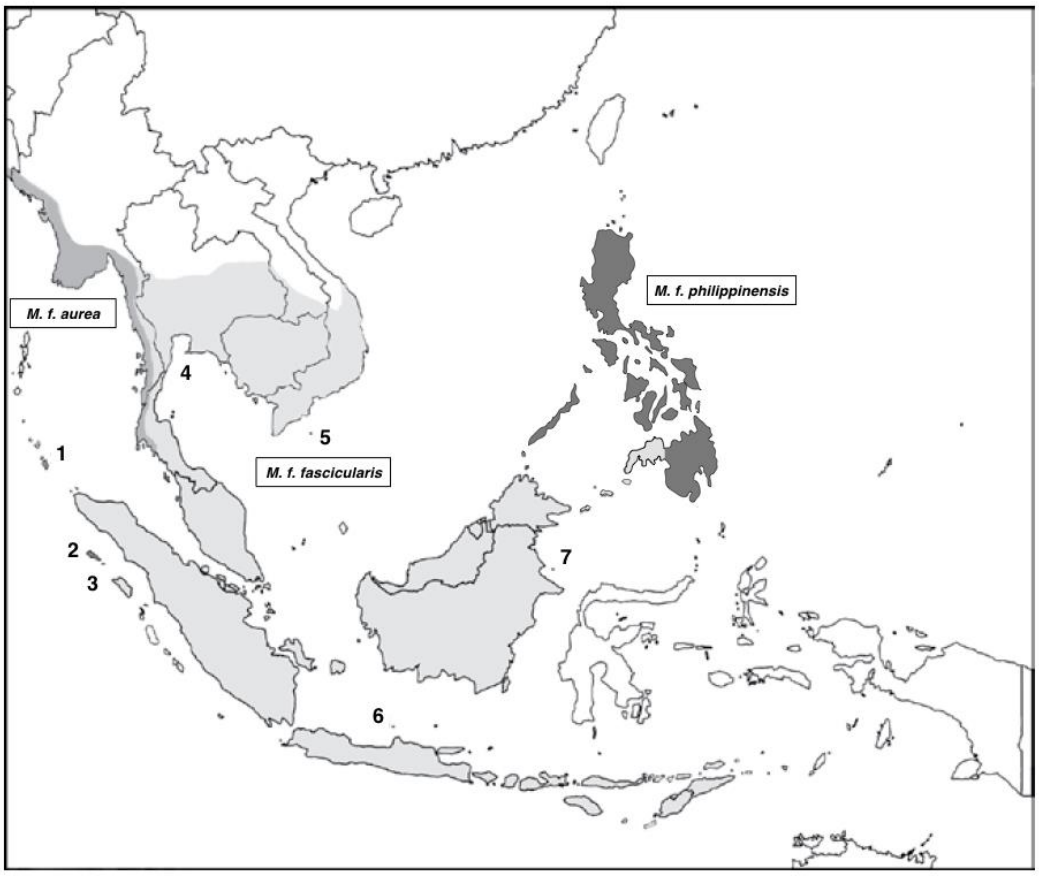

Figure 2. 3 The map showing the distribution range of *M. fascicularis*. The ranges of three main subspecies which are *M. facicularis aurea* (Burmese long-tailed macaque), *M. f. fascicularis* (common long-tailed macaque) and *M. f. philippinensis* (Philippine long-tailed macaque) are indicated by dark gray, light gray and black color. Other subspecies are labeled with number: 1 = *M. f. umbrosa*, 2 = *M. f. lasiae*, 3 = *M. f. fusca*, 4 = *M. f. atriceps*, 5 = *M. f. condorensis*, 6 = *M. f. karimondjawae*, 7 = *M. f. tua* (Adapted from Gumert, 2011).

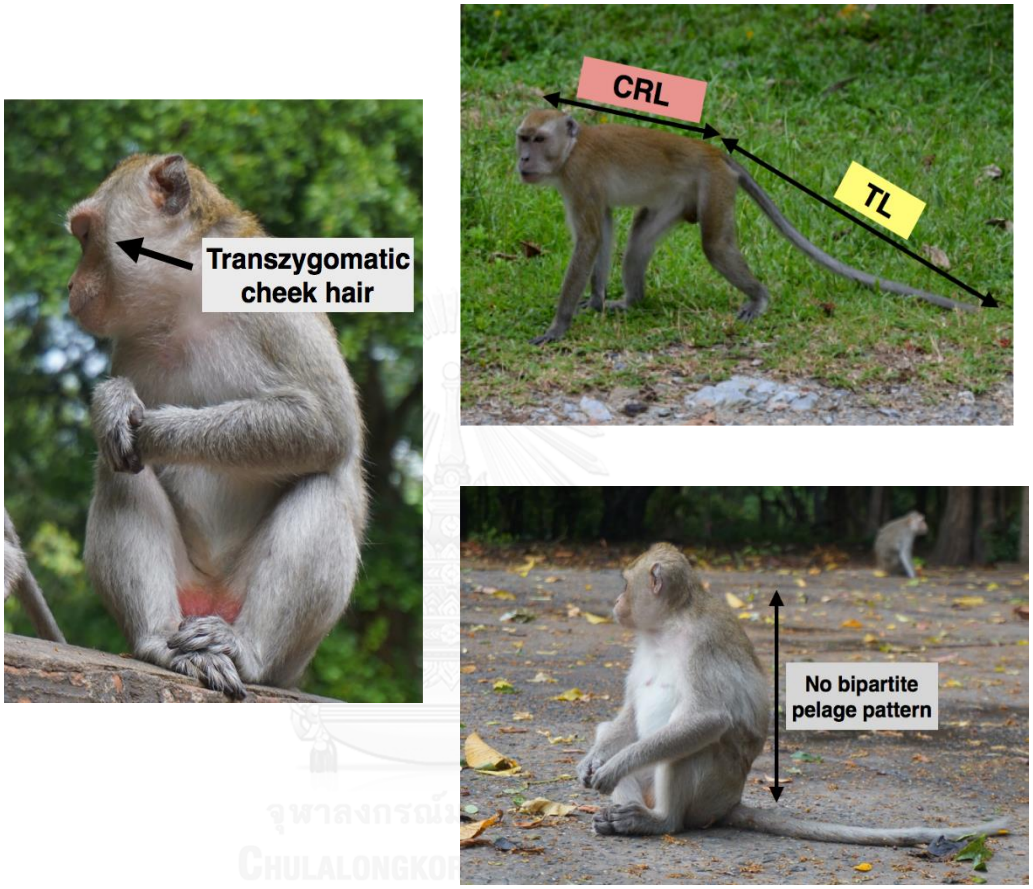

Figure 2. 4 Morphological characteristics of *M. fascicularis fascicularis*. TL and CRL stand for tail length and crown rump length, respectively.

2. Macaques in biomedical research

M. mulatta and *M. fascicularis* have been used as non-human primate models in biomedical research for more than half a century since the first primate breeding center, the Soviet Institute of Experimental Pathology and Therapy (IEPT), was established in 1923 (Johnsen, Johnson, & Whitney, 2012). One of the reasons for these species' use in research is that they have a wide range of distribution and were most commonly found. Moreover, their genetics are closely related to that of the humans; macaques share their last common ancestor with humans dating back to approximately 25 MYA (Kumar & Hedges, 1998; Glazko & Nei, 2003) while rodents and humans shares their last common ancestor approximately 60 MYA (Benton & Donoghue, 2007). Macaques and humans share 93.5% of their genetic identity (Gibbs et al., 2007), and as such, humans and macaques share many physiological, anatomical and immunological traits with each other (Kennedy, Shearer, & Hildebrand, 1997; Bontrop, 2001; Jerome & Peterson, 2001;). For example, female humans and macaques both exhibit a period of menopause which is unique among primate species. As such, macaques have been especially important in the study of reproductive senescence (Xenia, Joeph, & Barbara, 2005). In addition, manifestation of simian immune deficiency virus (SIV) infection in macaques is similar to human immunodeficiency virus (HIV) infection in humans, and as thus SIV is being used as a model for HIV vaccine development. In contrast, rodents have a different immune system causes them to be tolerant to this SIV or HIV infection (Sui et al., 2013). Comparisons of the rat and macaque brain reveal that the neocortex, which is the white matter in the brain, forms about 28% in rats while it is about 72% in macaques (Passingham, 2009). This has leads to the difficulty in interpretation based on studies using rats as a model for human brain research (Passingham, 2009). Thus, much of the translational research aimed at human biomedicine, such as development of drugs and vaccines for cardiovascular disease, neurodegenerative disease, infectious disease and bone disease, have been conducted in macaques. Currently, *M. mulatta* and *M. fascicularis* are the animals of choice for studying the SIV pathogenesis (Tsai et al.,

1994; Xue et al., 2013; Zhou et al., 2013), polio vaccine (Hörer et al., 2008; Sato et al., 2013), transplantation biology (Kean et al., 2006; Rood et al., 2007), diabetes mellitus (Arthur et al., 2013) and malaria (Anderios, Noorrain, & Vythilingam, 2010; Deye et al., 2012).

While the benefit of using these two species of macaques in biomedical research is significant, their inherent genetic variations are however of great concern. Heretofore, several studies revealed that these two species may show different responses across experiments. For example, *M. fascicularis* is the natural host for *Plasmodium cynomolgi* and *P. knowlesi*, (Zhang et al., 2017) thus they exhibited no severe manifestation of disease post infection while the infection in *M. mulatta* can cause fatal (Coatney et al., 1971; Carter & Gwadz, 1980). Motzel et al. (2003) found that *M. fascicularis* is also relatively resistant to tuberculosis (TB) infection compared to *M. mulatta*. A low dose of *M. tuberculosis* infection can cause both latent and active TB in *M. fascicularis* while causing progressive fatalities in *M. mulatta*. Due to the various types of manifestation of TB disease in *M. fascicularis*, in which ~40% of animals developed active TB and 60% developed latent infection, they have become the best model to represent the entire spectrum of human TB (Scanga & Flynn, 2014). Physiological parameters such as complete blood count, serum chemistry (Migot et al., 1999) and ABO blood groups (Moor-Jankowski & Socha, 1979) have also been demonstrated to be different in these two species.

In addition to species-specific differences, subspecies or regional differences can also influence the suitability of animal models for the study of any diseases. Because of *M. mulatta*'s wide range of distribution, its genetics reflects the geographic distance and/or geographical barriers among the different regional populations of this species. For example, Chinese *M. mulatta* can maintain higher $CD4^+$ lymphocyte levels after being infection by SIV compared to *M. mulatta* from India (Trichel, Rajakumar & Murphey-Corb, 2002). Therefore, Indian *M. mulatta* are more susceptible, and thus

considered more suitable for the SIV studies than their conspecific from China (Joag et al., 1994).

3. Hybridization scenario between *M. mulatta* and *M. fascicularis* in their natural habitats

Natural hybridization affects primate evolution by means of the formation of new taxa and the genetic enrichment through introgressive hybridization (Arnold & Meyer, 2006). A prominent evidence is the emergence of *M. arctoides* which is likely a result of the hybridization either between the ancestors of *mulatta* and *sinica* species groups (if macaques are categorized into seven species groups following Zinner et al., 2013) or between *fascicularis* and *sinica* species groups (if macaques are categorized into four species groups following Fooden, 1976). More recent case was found in Sulawesi macaques when two species of them, *M. maura* and *M. tonkeana* were reported as hybrids on southwestern Sulawesi, Indonesia (Evan, Supriatna, & Melnick, 2001). In addition to natural hybridization in macaques, there is also human- assisted hybridization. For instance, the release of *M. nemestrina* (pig-tailed macaque) and *M. mulatta* into *M. fascicularis* troops at Kumpawaphi City Park and Khao Khieow Open Zoo, respectively, have resulted in hybrid animals. (Malaivijitnond & Hamada, 2008; Jadejaroen et al., 2016).

As described earlier, the distribution range of *M. mulatta* is *ca.* 15 to 36° N which has mainly subtropical climate, while *M. fascicularis* spread throughout mainland and island in Southeast Asia *ca.* 20° N to 10° S which is within the tropical zone. Based on these species distribution ranges, Fooden (1995, 2000) and Hamada et al. (2006) described the parapatric zone between the two species at around northern Indochina (15-20° N) covering Myanmar, Thailand, Laos and Vietnam, from west to east, respectively. As *M. mulatta* and *M. fascicularis* possess similar characteristics of glans penis, they were categorized into the same species group by Fooden (1976), it was deduced that the

interspecific hybridization is possible between these species. Additionally, the hybrid offspring of these macaques are fertile and can back cross with either to the full-blood *M. mulatta* or *M. fascicularis* that can lead to a broad range of genetic admixture beyond the proposed hybrid zone. Therefore, the natural hybridization between these two macaque species has become a point of interest both in the fields of biomedical and evolutionary studies over the past decade. Several studies on hybridization between these species based upon morphological, physiological, and genetic analysis have been performed. (Hamada et al., 2006; Malaivijitnond, Sae-Low, & Hamada, 2008; Tosi et al., 2002; Jadejaroen et al., 2015; 2016). Tosi et al. (2002) hypothesized that the hybridization between *M. mulatta* and *M. fascicularis* was driven by the introgression of male *M. mulatta* into *M. fascicularis* populations during the Pleistocene epoch (Osada et al., 2010). The mtDNA phylogeny reveals a clear separation between *M. mulatta* and *M. fascicularis* lineages forming monophyletic patterns for each clade. Nevertheless, Y-chromosome phylogeny showed incongruent phylogenetic patterns; Indochinese *M. fascicularis* were separated from the Sundaic *M. fascicularis*, but grouped with *M. mulatta* resulting in paraphyly of *M. fascicularis*. Hamada et al. (2006) collected morphological data, for instance, body mass and proportion, RTL and pelage colors of *M. mulatta* at Loei province, northeastern Thailand (ca. 17° N), which was situated in the hybrid zone between *M. mulatta* and *M. fascicularis*. Their results supported the hybridization hypothesis of which the morphological characteristics of these Thai *M. mulatta* were intermediate between those of *M. mulatta* in China and India and those of *M. fascicularis* in Thailand. The study of human-ABO blood group in Thai *M. mulatta* and *M. fascicularis* also supported the hybridization hypothesis (Malaivijitnond et al., 2008). Interestingly, Thai *M. mulatta* possessed all four types of ABO blood group and the frequency were B = 0.396, AB = 0.334, A = 0.182 and O = 0.088 which are more similar to those of Thai *M. fascicularis* that showed the frequency of AB = 0.296, O = 0.274, B = 0.272 and O = 0.158, but are quite different from their conspecifics in China

and India who owed only A, B and AB blood group at the frequency equal 0.21, 0.51 and 0.28, respectively (Premasuthan et al., 2011).

Although there have been many studies on hybridization between these two species for more than a decade, some issues, for examples; the limited area and direction of genetic introgression, are still obscure. This is probably because results from those studies are largely from captive nonhuman primate populations. Some studies have suggested that the unidirectional gene flow form male *M. mulatta* to *M. fascicularis* was restricted near the Isthmus of Kra (Tosi et al., 2002; Bonhomme et al., 2009, Steviosn & Kohn, 2009). Other studies have however argued against this scenario and proposed bi-directional hybridization events where gene flow can either stem from *M. mulatta* to *M. fascicularis* or vice versa (Kanthaswamy et al., 2010; Osada et al., 2010; Hamada et al., 2016). Some have also proposed that *M. mulatta* gene flow was extended far beyond Indochina and southward toward Sundaland, while the gene flow from *M. fascicularis* to *M. mulatta* expanded more northward into eastern China and India (Kanthaswamy et al., 2010; Osada et al., 2010).

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4. Biogeography in Thailand

Thailand is a hotspot for biodiversity as it encompasses two different biogeographical regions; Indochina and Sunda. The Indochinese bioregion refers to mainland Southeast Asia including Myanmar, Vietnam, Laos, Cambodia, and Thailand except for its southern peninsular, while Sundaic bioregion includes the southern peninsular of Thailand, Peninsular Malaysia and Sumatra, Java, Borneo, Bali, and Lesser Sunda Islands until Timor Island. Wallace (1876) first suggested that the transition zone between these two bioregions located near the Isthmus of Kra, around 12-13° N (Figure 2.5). However, In 1976, Wells, whose studies were based on bird species in those regions found that the transition zone is probably locates north of the previous proposed area (Wallace, 1876) which near the Isthmus of Kra, at 10° 30′ N. This proposal was later supported by many other studies (Bunsong & MacNeely, 1988;

Inger, 2001; Hughes et al., 2003). However, another transition zone, the Kangar-Pattani line, which was well-known as the boundary between Indochinese-Sundaic plants among botanists was also proposed (Figure 2.5). This Kangar-Pattani line is located approximately 500 km further south of the Isthmus of Kra (6–6° 5´ N) and corresponded with the transition area between perhumid evergreen rainforest and the wet seasonal evergreen rainforest (van Steenis, 1950). This hypothesis was also supported by animal distribution patterns observed in many studies (Woodruff & Turner, 2009; Hughes et al., 2011). Interestingly, Malaivijitnond et al. (2012) surveyed the distribution ranges of pig-tailed macaque and proposed that the zoogeographical barrier that separated these macaque species into northern (*M. leonina*) and southern (*M. nemestrina*) populations was along the Surat thani-Krabi depression (~8-9° 30´ N), an area between Isthmus of Kra and Kangar-Pattani line. A previous study by Hughes (2011) determining bat distribution from 20° N to the southernmost of Peninsular Malaysia, covering Indochina and Sunda regions, they found two major transitional zones at 6-6° 5´ N and 13-13° 5´ N, and one smaller peak at 11° N for various bat species.

In fact, climate fluctuation in the Pleistocene epoch may have also had an impact on animal migration, and thus may have shifted the boundary between Indochinese and Sundaic regions northwardly or southwardly which probably led to the gradual changed on species distribution around these regions (Hughes et al., 2011; Tougard, 2001). Besides the main zoogeographical barriers mentioned earlier, Thailand also has several additional biogeographical barriers such as the Taninthanyi range, the Phuket range as well as the Nakhon Si Thammarat mountain which could also potentially have an effect on animal distribution.

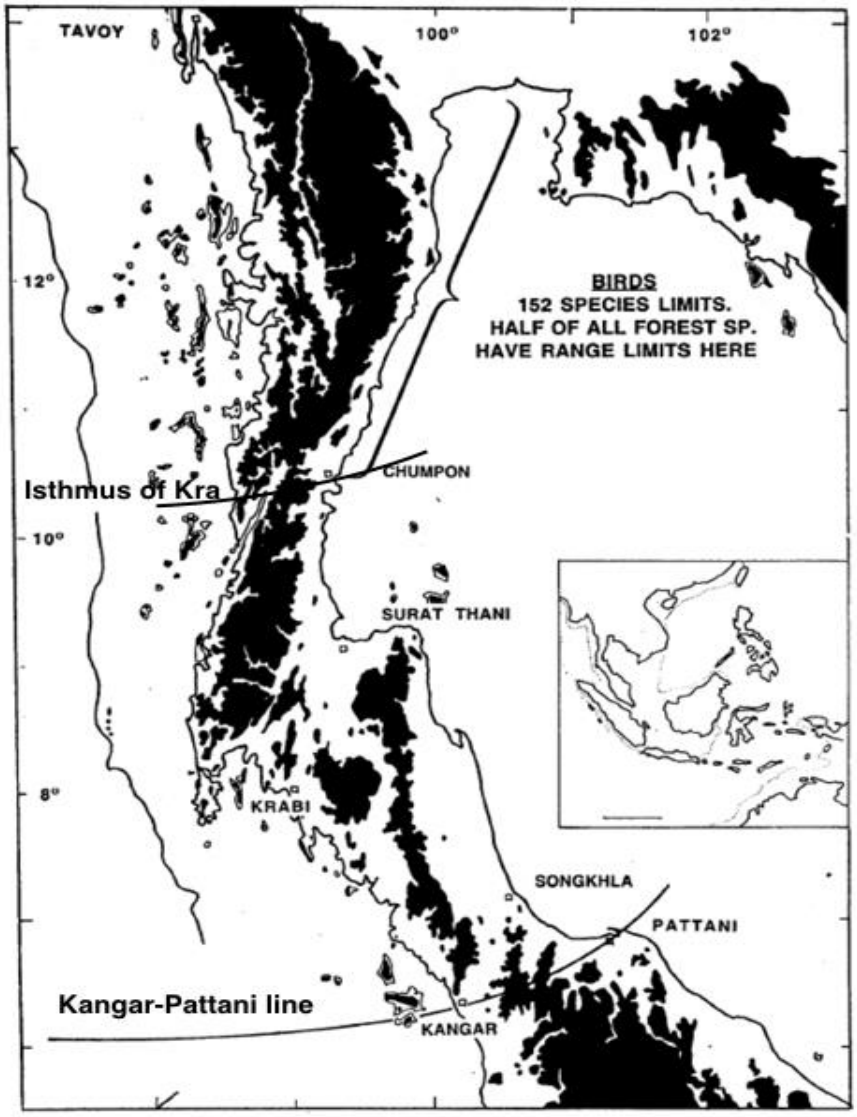

Figure 2. 5 The map shows two main zoogeographical barriers; Isthmus of Kra and Kangar-Pattani line. (Adapted from Woodruff, 2003)

5. Geographical changes during the Pleistocene epoch

The Pleistocene is one of the geological epoch ranging from 2.588-0.012 MYA (Gibbard et al., 2010). It was the time of intensive climatic fluctuations which were repeated between glacial (cold) and interglacial (warm) periods. Richmond & Fullerton (1986) reported that more than 11 major glacial events and many of minor events were occurred in the United States. In the last glacial maximum (LGM), at which the end of this period corresponds with the end of Pleistocene epoch, was the time that massive ice sheets covered many areas in North America, northern Europe, and Asia. As such, most of the areas on earth was cold and dry. The formation of ice sheet at the northern hemisphere reduced global sea level up to 120 meters at approximately 21,000 year ago (Figure 2.6-2.12) and formed continental shelves in many areas such as Sunda shelf connecting between Indochina and Sunda regions (Sathiamurthy & Voris, 2006) and Sahul shelf connecting between New Guinea, Australia and Tasmania (van Andel & Veevers, 1965; Harrison et al., 2006) (Figure 2.13).

Sundaland comprises Sumatra, Java, Borneo, Palawan, Bali, the Mentawai Islands, and southern Asian mainland. The eastern boundary of Sundaland was restricted by Wallace's Line which runs through the Philippines (between Palawan and Luzon) and southward through Indonesia (between Borneo and Sulawesi, and between Bali and Lombok) which was defined as the boundary of Oriental zoogeographic province (Huxley, 1868; Simpson, 1977). Because of the emersion of landmass during the glacial period, many previous studies have characterized Sundaland as the area in which most animal species could freely migrate (Heaney, 1985; Kahlke, 1972; Abdul-latiff et al., 2014a). However, most of the areas was cooler and drier than the present time, together with longer in dry season and shorter in wet season resulting in more pronounced seasonality (Heaney, 1991). The forest area mainly remained on the Sundaic region (southern area), whilst the Indochinese region (northern area) was largely covered by savanna (Heaney, 1991; Meijaard, 2003) (Figure 2.14). Repeated fluctuation in

climate, vegetation types, and sizes of landmass during the glacial and interglacial periods in the Pleistocene epoch led to the expansions or contractions of habitat types, changes in macroclimate and microclimate, and thus affected the distribution of animal species together with the extinction and colonization rates.

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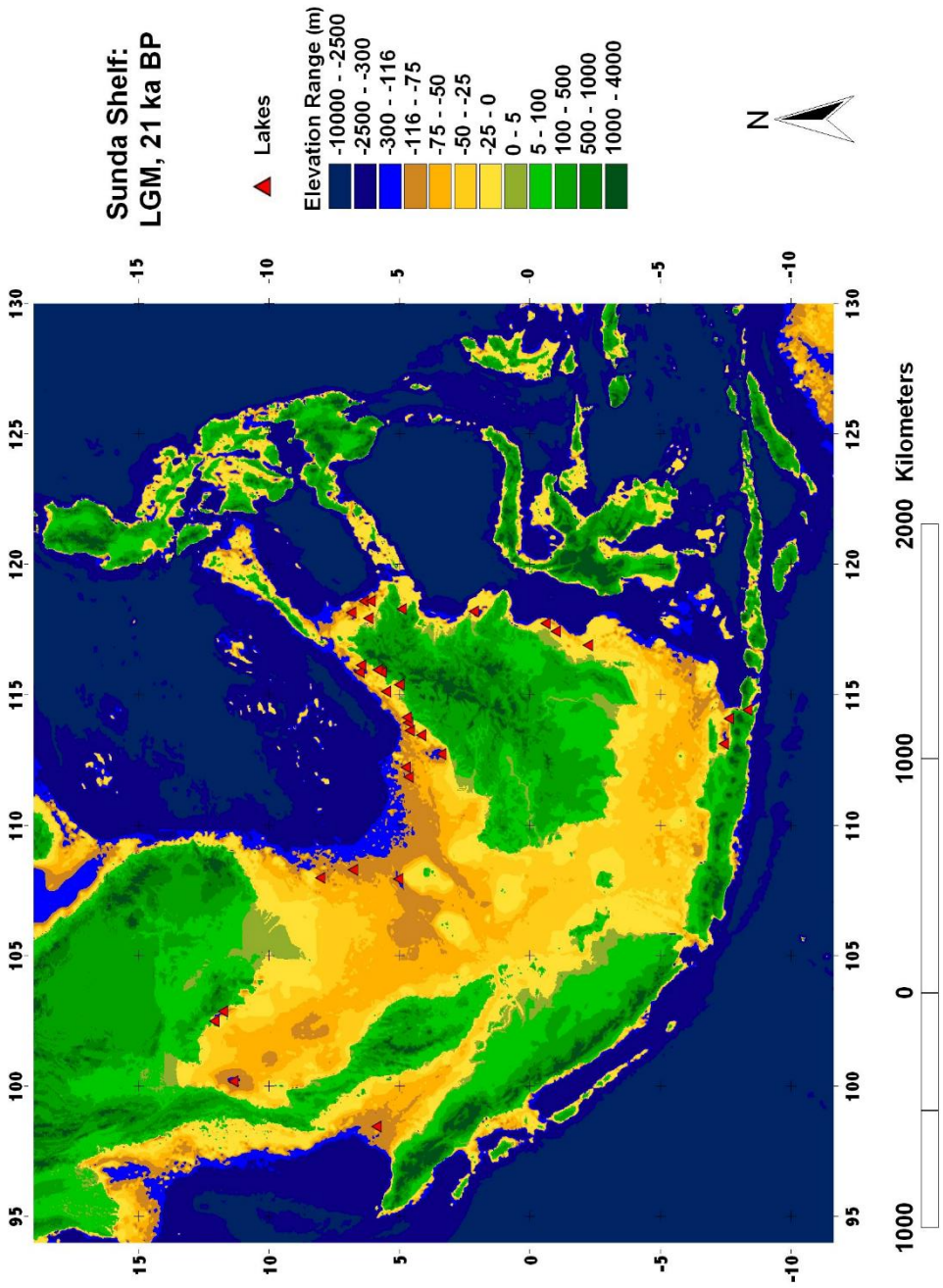

Figure 2.6 Sunda shelf during last glacial maximum (LGM) at 21 ka when the sea level dropped up to 116 meters below the present-day sea level (Source: Sathiamurthy & Voris, 2006).

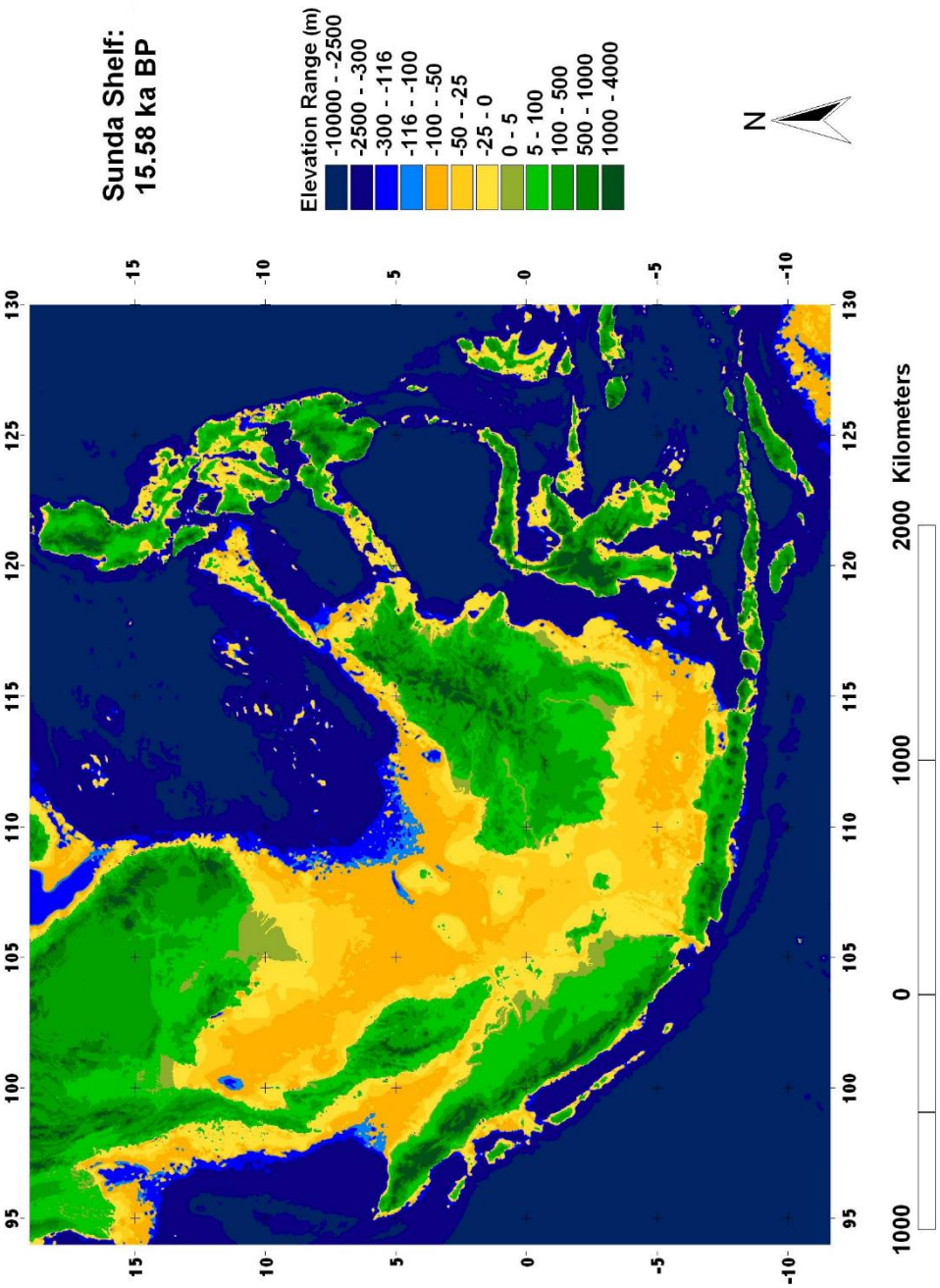

Figure 2. 7 Sunda shelf during last glacial maximum (LGM) at 15.58 ka the sea level dropped up to 100 meters below the present-day sea level (Source: Sathiamurthy & Voris, 2006).

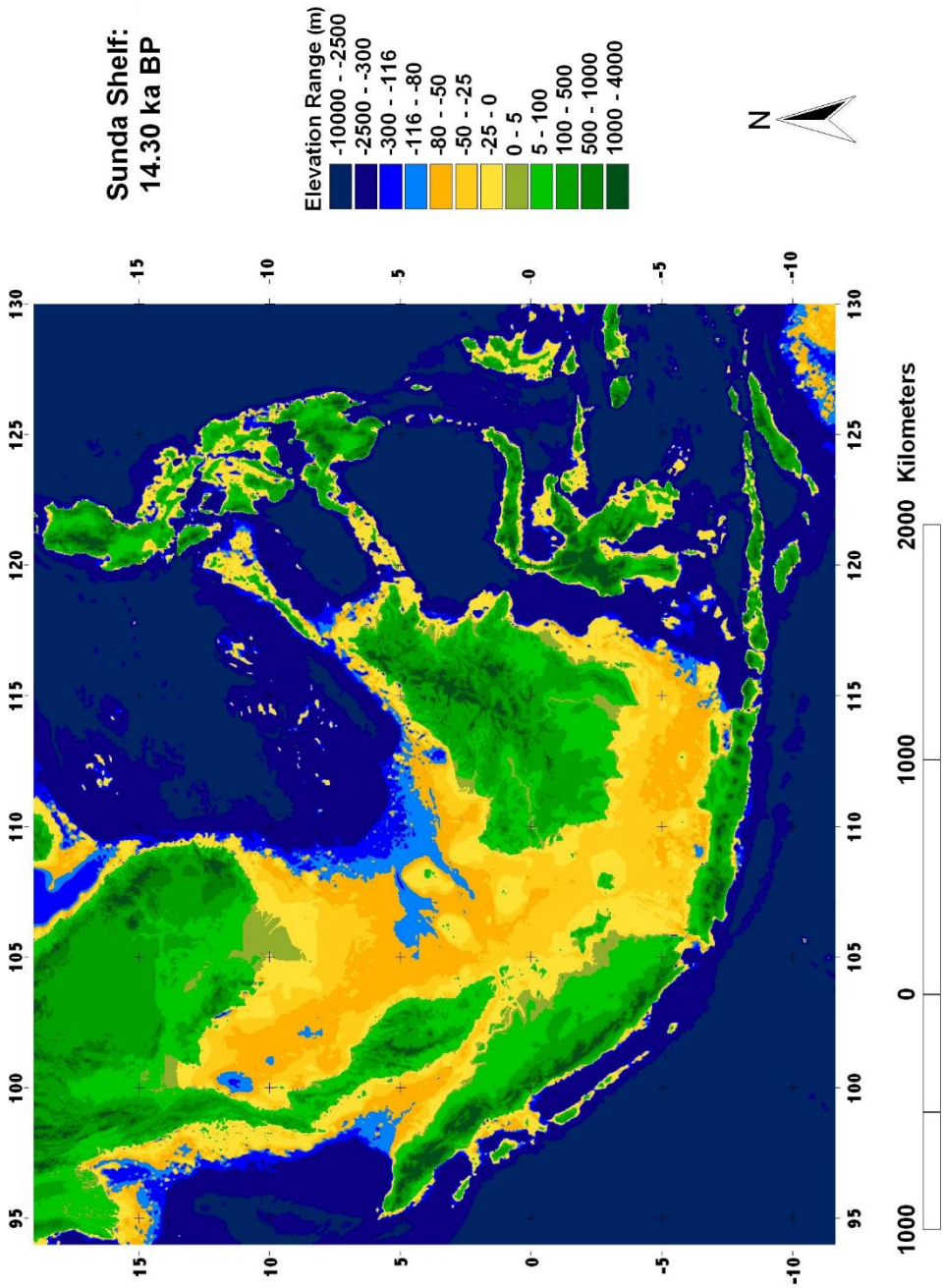

Figure 2.8 Sunda shelf during last glacial maximum (LGM) at 14.30 ka when the sea level dropped up to 80 meters below the present-day sea level (Source: Sathiamurthy & Voris, 2006).

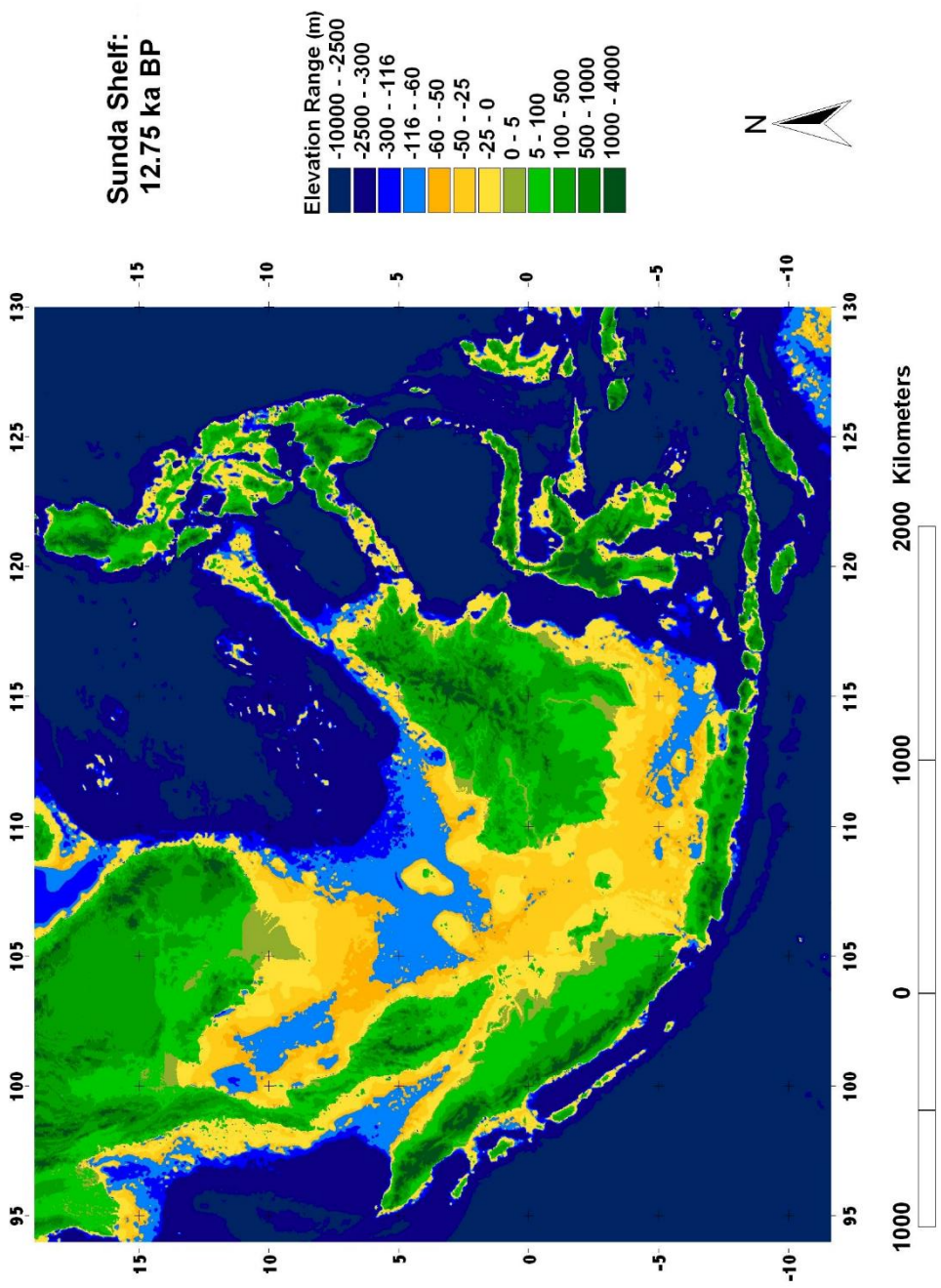

Figure 2. 9 Sunda shelf during last glacial maximum (LGM) at 12.75 ka when the sea level dropped up to 60 meters below the present-day sea level (Source: Sathiamurthy & Voris, 2006).

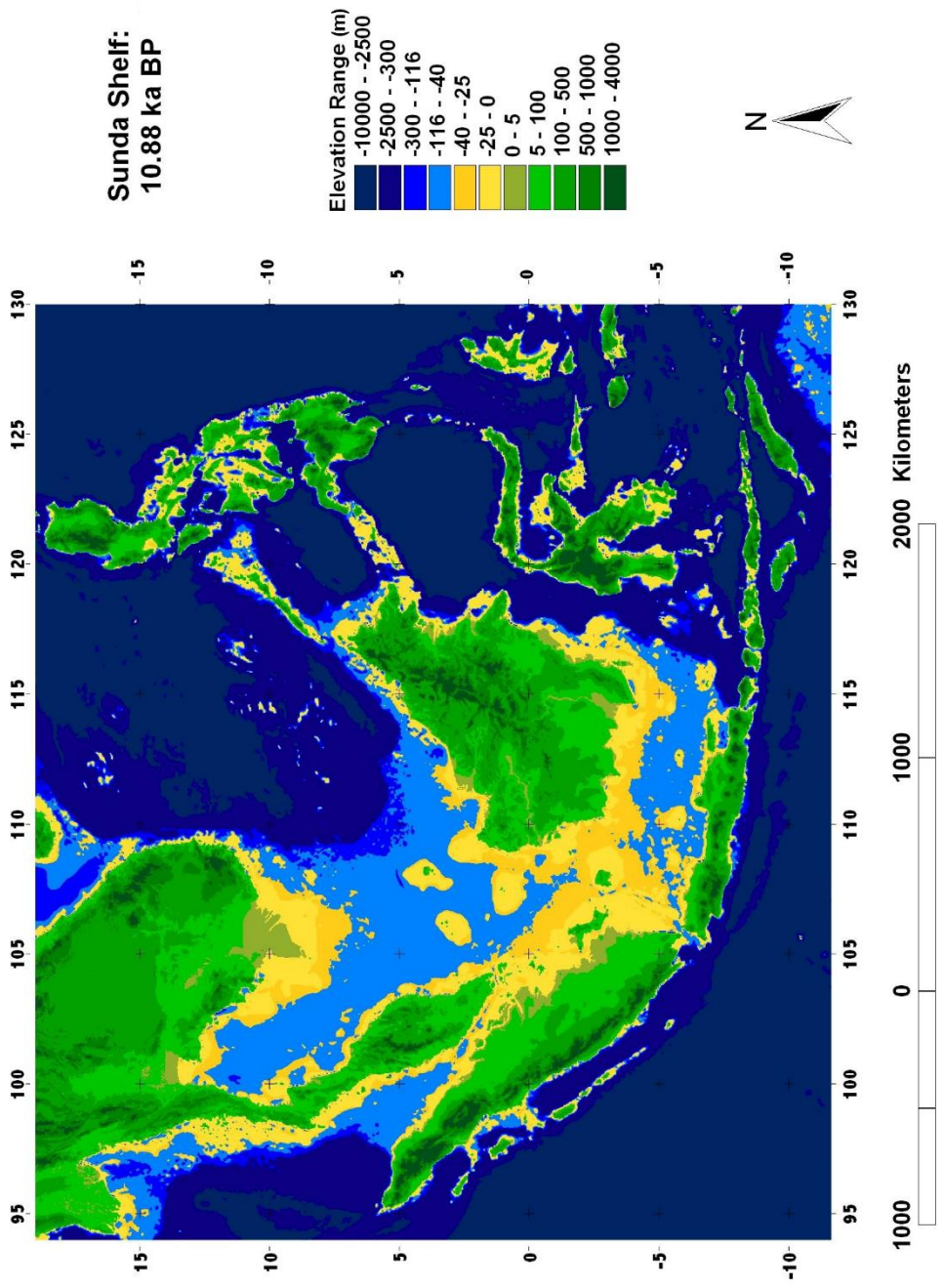

Figure 2. 10 Sunda shelf during last glacial maximum (LGM) at 10.88 ka when the sea level dropped up to 40 meters below the present-day sea level (Source: Sathiamurthy & Voris, 2006).

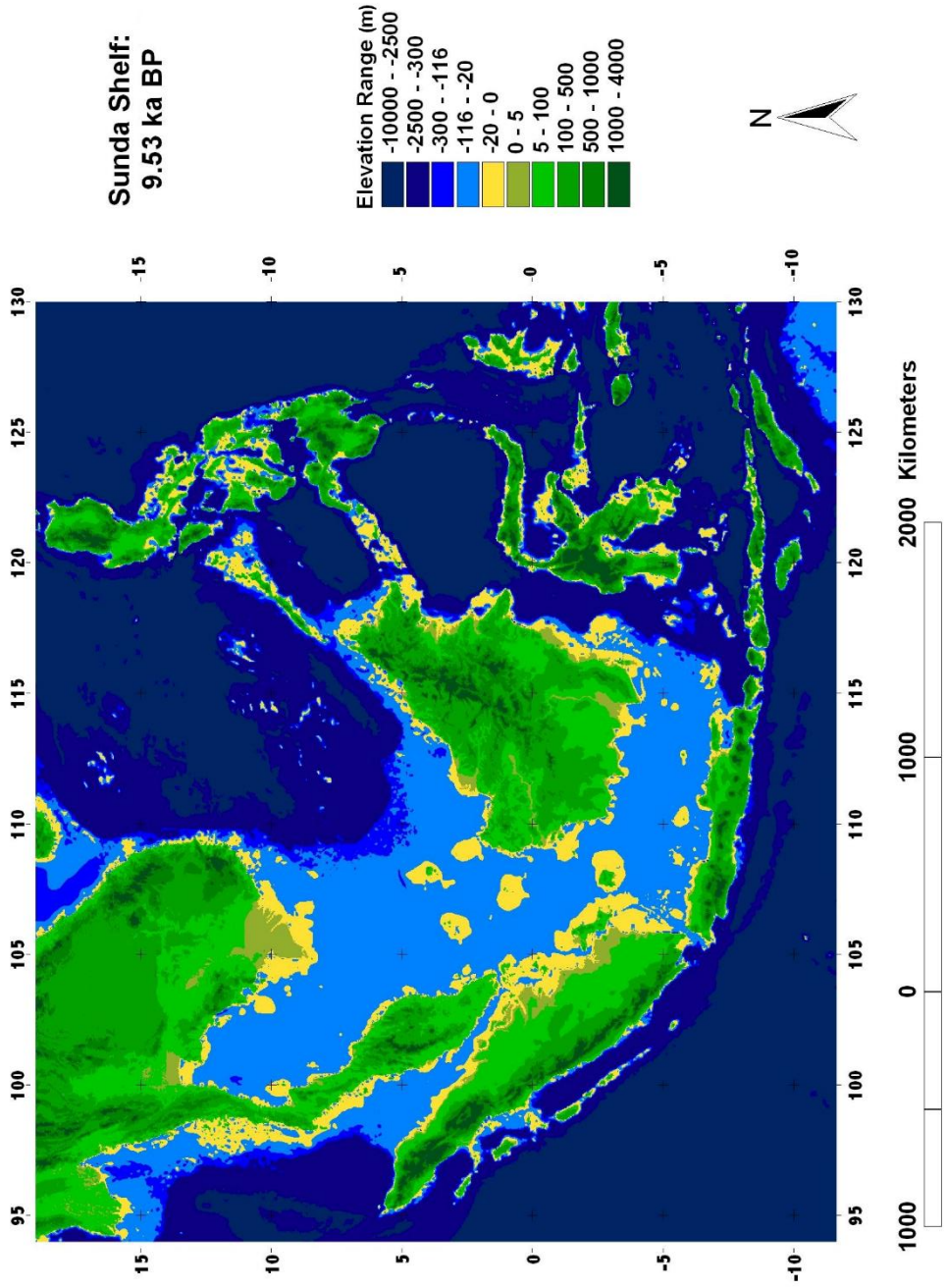

Figure 2. **11** Sunda shelf during last glacial maximum (LGM) at 9.53 ka when the sea level dropped up to 20 meters below the present-day sea level (Source: Sathiamurthy & Voris, 2006).

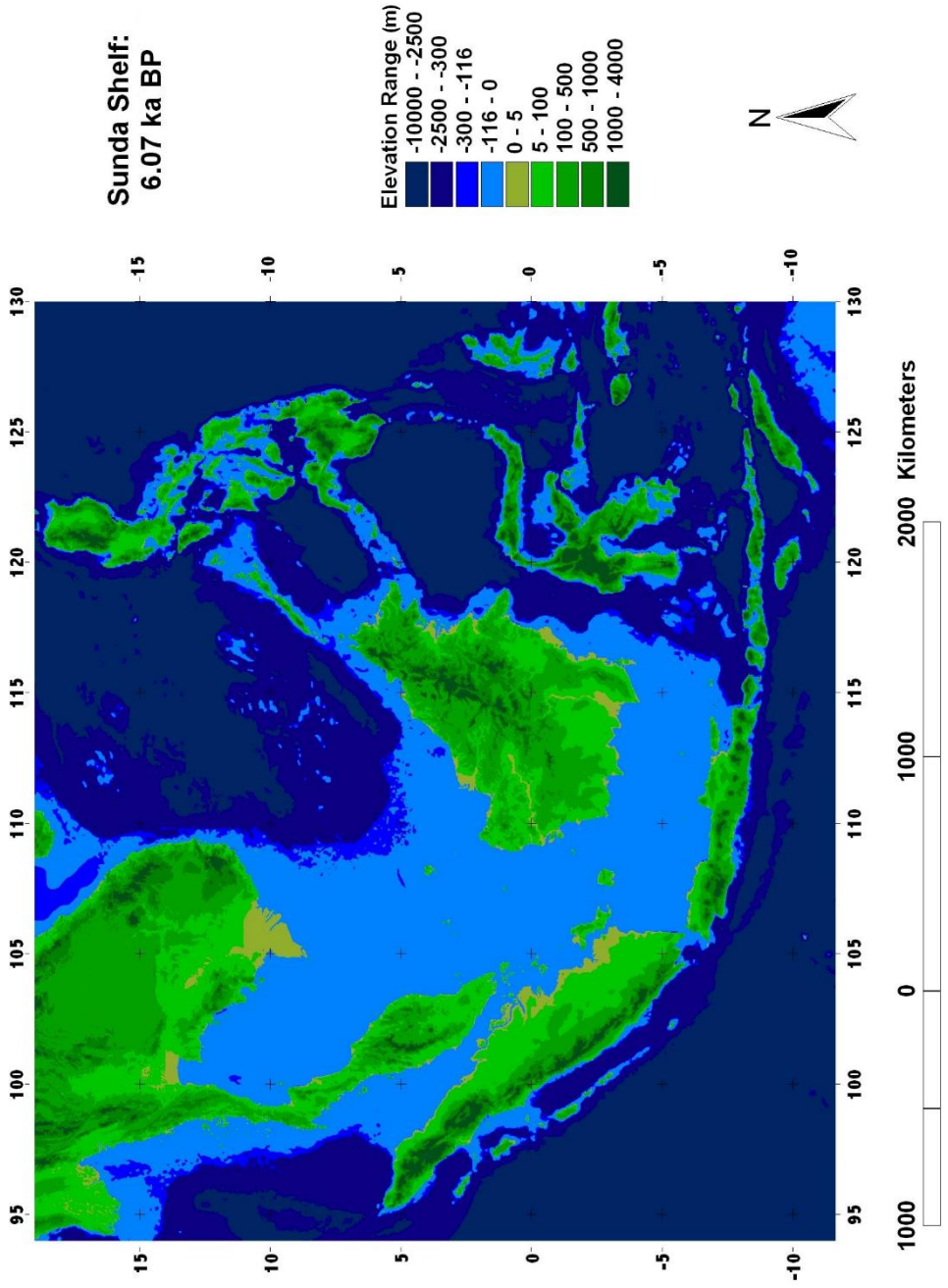

Figure 2. 12 Sunda shelf during last glacial maximum (LGM) at 6.07 ka when the sea level equal to the present-day sea level (Source: Sathiamurthy & Voris, 2006).

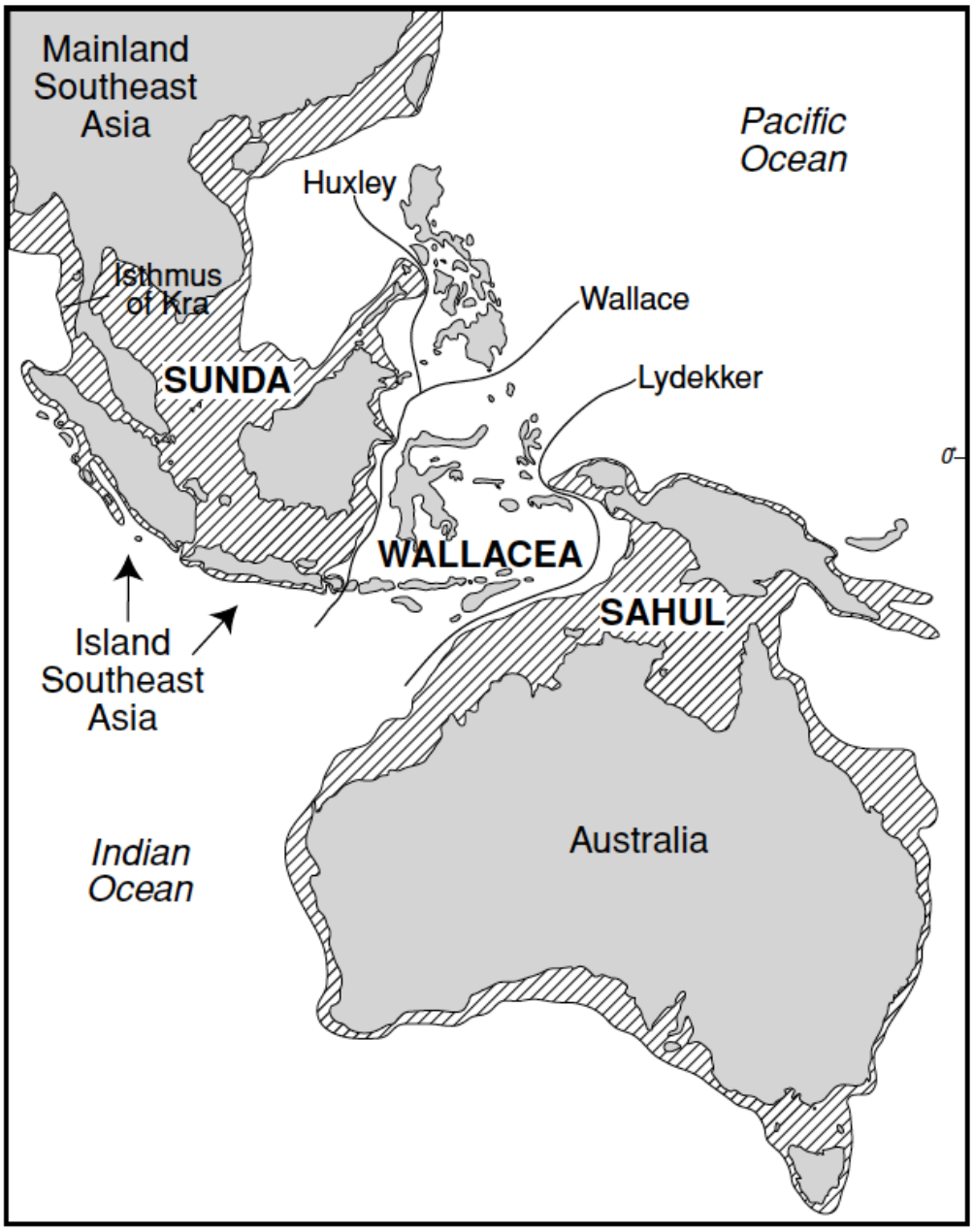

Figure 2. 13 The map shows Sunda and Sahul shelf (Source: Harrison et al., 2006)

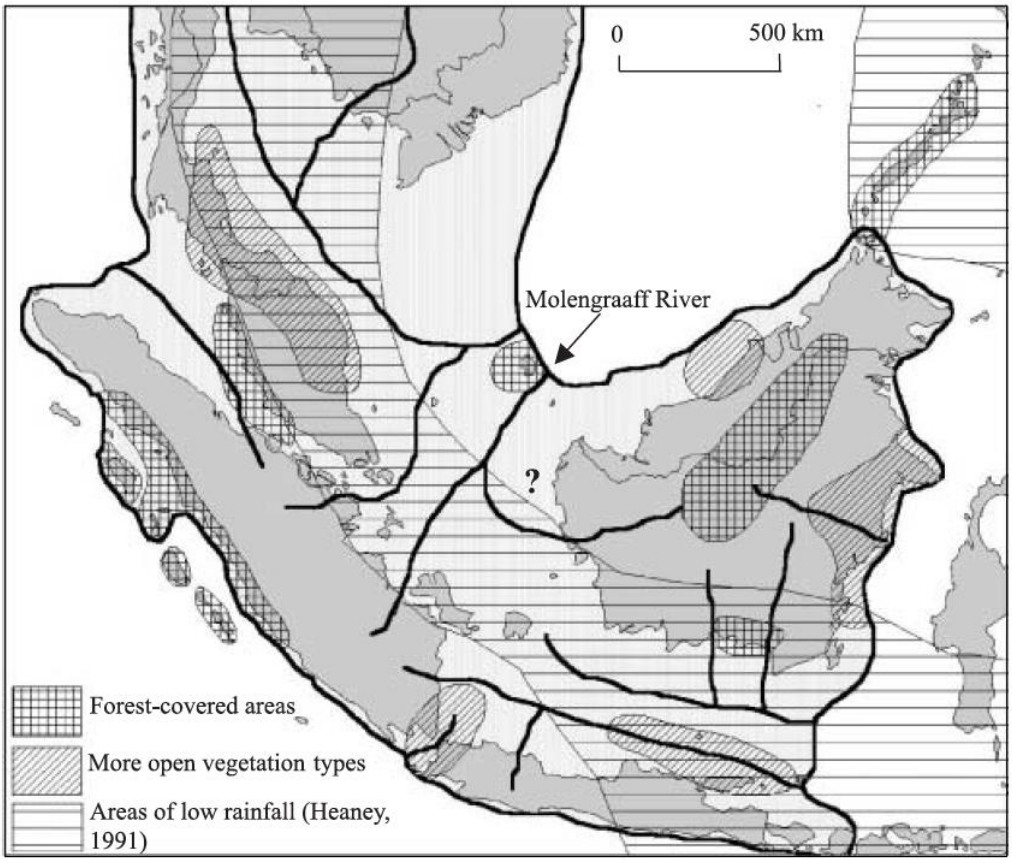

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Figure 2. **14** Forest types during the last glacial maximum (LGM) (Source: Meijaard, 2003)

6. Genetic markers for population and hybridization assessments

Considering the social organization of macaques, females usually lived permanently in their natal group (female philopatry), but the males tend to migrated out of the group when they became mature (male fission). Based on this social structure, the examination of the role and importance of natural hybridization in the evolutionary histories of these macaque species should be carried out using maternally inherited, sex-linked and autosomal genetic markers such as mtDNA, Y chromosomes, autosomal SNPs as well as STRs, respectively.

6.1 Mitochondrial DNA

The mitochondrion is one of several vital organelles in eukaryote organisms as it plays an important role in energy production. Unlike any other organelles, mitochondrion has its own genomic DNA located within its matrix, i.e., mitochondrial DNA (mtDNA; Taanman, 1999). This circular genome was thought to have been derived from bacteria which incorporated into an ancestor of the eukaryotic cell during its evolution (Gray et al., 1999). mtDNA is restricted only in female germ line in most of multicellular organisms, and as such there are two suggested mechanisms underlying the maternal inheritance. Simple dilution model is the hypothesis in which the lower copy numbers of paternal mtDNA are diluted away by the excess copy of oocyte mtDNA. Another model is active degradation model in which the paternal mtDNA is thought to be selectively degraded, either before or after fertilization (Sato & Sato, 2013). These mechanisms have led to elimination of the paternal mtDNA and preventing it's transmission to the offspring. Based on its maternal inheritance logic without genetic recombination, mtDNA is generally used as a matrilineal marker in macaque evolutionary studies.

Up to now, mitochondrion sequence studies were performed in many macaque species, including *M. mulatta*, *M. fascicularis*, *M. arctoides*, *M. cyclopis*, *M. Sylvanus*, *M. silenus* (lion-tailed macaque), *M. fuscata* and *M. thibetana* (Thibetan macaque) (Liedigk et

al., 2014; Gokey et al., 2004; Huang et al., 2015; Wang et al., 2016). Basically, the mtDNA is approximately 16,560 bp in length and it encodes 13 proteins, 2 ribosomal RNAs, 22 transfer RNAs and 1 control region (D-loop) (Huang et al., 2015; Wang et al., 2016) (Figure 2.15). Among these mtDNA structures, the hypervariable segment I (HVS1) on D-loop can be used to distinguish between closely related species and subspecies of macaques because of its highly polymorphic property (Smith & McDonough, 2005; Smith, McDonough, & George, 2007).

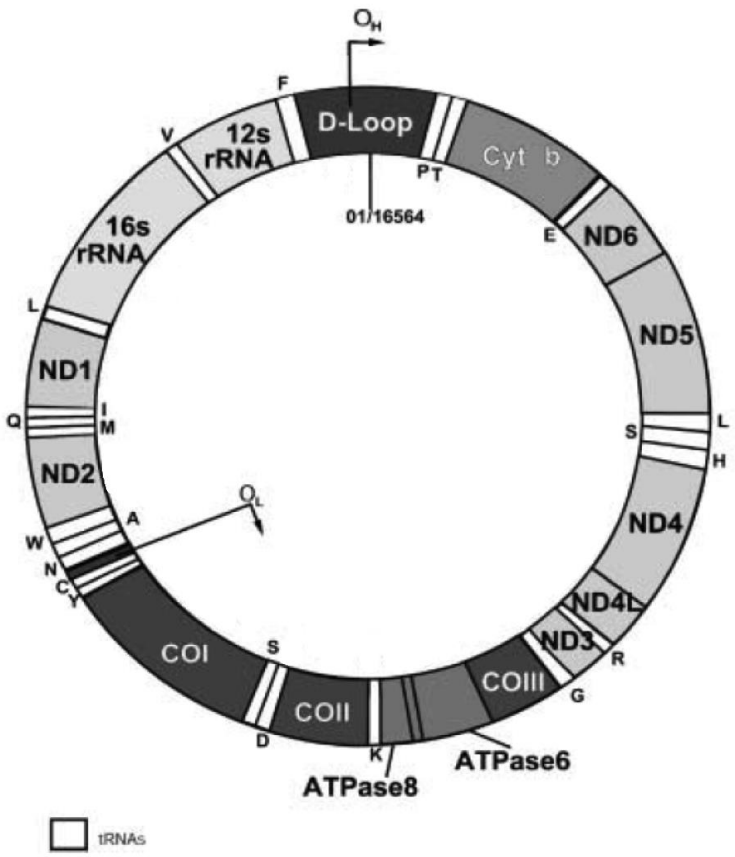

Figure 2.15 Mitochondrion sequence of *M. mulatta* (Adapted from Gokey et al., 2004).

6.2 Y-chromosome-linked genes

Y-chromosome is one of the two sex chromosomes, apart from the X chromosome, found in mammals and many animal species. Both sex chromosomesare thought to be derived from an autosomal chromosome. The original Y-chromosome is thought to have experienced genetic decay owing to either an accumulation of deleterious mutations at ancestral genes or a lower rate of adaptation relative to the X-chromosome (Bachtrog, 2013) (Figure 2.16).

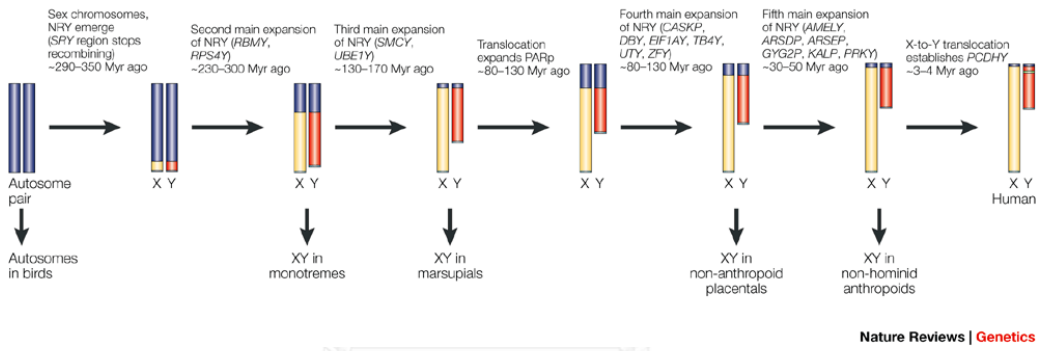

Figure 2. 16 The origin of the mammalian Y-chromosome (Source: Lahn, Pearson & Jegalian, 2001).

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Although the Y-chromosome has subsequently evolved independently across multiple groups of animals and plants, all Y-chromosomes have shared two unique characteristics: they are restricted only in male germ line and also lack recombination with homologous regions over most of its length (except the presudoautosomal region (PAR) where recombination takes place between the Y and X chromosomes). The main compartment on the Y-chromosome is called male-specific region (MSY) which constitutes up to 95% on the chromosome; the remaining 5% is the PAR (Skaletsky et al., 2003). Despite its importance, only a handful of the Y-chromosome sequences of

primates such as human, chimpanzee, *M. mulatta* and *M. fascicularis*, have been completely sequenced to date (Skaletsky et al. 2003; Hughes et al., 2010, 2012). The most challenging step in completing whole sequence analyses of Y-chromosome is the assembly of the highly repetitive regions and those of palindrome sequences within this chromosome (Tomaszkiewicz et al., 2016).

The comparison of Y-chromosomes between three primate species, human-chimpanzee-*M. mulatta* has revealed the differences in their sizes and components across primate taxa (Hughes et al., 2012). Humans and chimpanzees have the heterochromatinized regions near the centromere while the *M mulatta* do not. Moreover, the euchromatic segment of this chromosome which mainly comprises X-chromosome degenerate and Ampliconic regions in *M mulatta* is notably smaller compared to those of humans and chimpanzees. However, the location of PAR in *M. mulatta* corresponds to those of chimpanzees and humans (the short-arm PAR) (Hughes et al., 2012) (Figure 2.17).

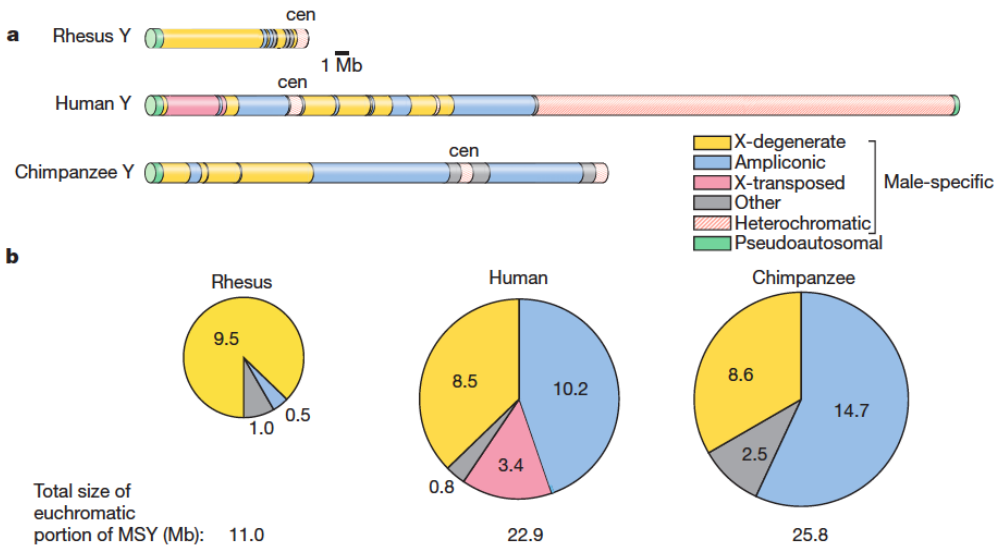

Figure 2.17 Comparison of the Y-chromosomes among human, chimpanzee and *M. mulatta* (Source: Hughes et al., 2012).

Several genes on the Y-chromosome, including the sex-determining region (*SRY*) and the testis specific, Y-chromosome (*TSPY*) genes were subjected for macaque evolutionary studies in the past decade (Tosi, Morales, & Melnick, 2000, 2002; Tosi & Coke, 2007). *SRY* is crucial for the initial step of male sex determination while *TSPY* plays an important role in spermatogenesis.

6.3 Short tandem repeats (STRs)

Short tandem repeats (STRs) which are also known as microsatellites or simple sequence repeats (SSRs) are the tandem repeat of nucleotides between 2 to 7 base pairs (bp) in approximately a half dozen to several dozen times (Butler, 2007). According to the length of repeated unit, they are classified into di-, tri-, tetra-, penta-, hexa- and hepta-nucleotide repeats. Basically, the name of STR refers to the provenance of each locus. For example, locus D1S548, D stands for DNA, 1 indicates the number of chromosome which is 1 in this case, S stands for STR, and 548 is the unique identification.

STRs are found in both prokaryote and eukaryote along their genomes. The 3% of human genomes are accounted for STRs which mostly found in non-coding regions (Fan & Chu, 2007). Although the distribution of STRs is not uniform, in average, STRs are found in every 2,000 bp (Cullen et al., 2003). The mutation rate of STRs ranging from 10^{-5} to 10^{-2} nucleotide/generation (Di Rienzo et al., 1994) which is much higher than the normal DNA sequence which occurred approximately 10^{-9} nucleotide per generation (Alberts et al., 2002). To date, three hypotheses have been proposed about the mechanisms of STR mutation. Firstly, unequal crossing over in meiosis, this mechanism generates a large block of satellites DNA by exchange the repeated unit between homologous chromosomes. However, because the crossing over plays an important role between different chromosomes, this mechanism plays a restricted role in STR mutation (Fan & Chu, 2007). Secondly, retrotranposition mechanism, Nadir et al. (1996) found that most A-rich STRs are generated by a 3'-extension of retrotranscripts, which

is similar to that of polyadenylation of mRNA. However, a high density of transposable elements does not always conform with a high density of STRs, thus further studies are still needed to uncover this mechanism (Fan & Chu, 2007). The last hypothesis is strand-slippage replication which was regarded as the main pattern of STRs mutation (Jobling et al., 2013). The slippage occurs when DNA polymerase skips or copies one or more repeating units during the DNA replication resulting in a mispairing between the nascent and template strands. Subsequently, this mismatch formed a loop and altered the repeated number of the STRs when they are used as the template in next cycle of DNA synthesis.

Although most of STRs are regarded as junk DNA because they have no biological function, several hypotheses are raised to explain their role in an organism. For example, Streelman & Kocher (2002) found the association between the differences in number of (CA/GT)n microsatellite in *prolactin 1* (*prl 1*) promoter and the expression of *prl 1* gene which contribute to the growth response and salt tolerance in tilapia fish species. Liu et al. (2016) found that STRs are significantly overrepresented in the gene involved in pathways such as Ubiquitin mediated proteolysis, RNA degradation, Spliceosome and Terpenoid backbone biosynthesis.

Because of their polymorphisms and high mutation rate, STRs play an important role as a marker in various genetic studies such as population genetics, paternity testing, and molecular breeding. Currently, several STR markers have been developed and popularly used among primate species including macaques (Bonhomme et al., 2009; Kanthaswamy et al., 2013; Smith et al., 2014; Sukmak et al., 2014).

6.4 Single nucleotide polymorphisms (SNPs)

Single nucleotide polymorphism or SNPs (pronounced "snip") is a single nucleotide variation in DNA sequences which occurred more than 1% in a population. SNPs are the most common type of genetic variation among individuals in population. Because SNPs occur throughout the genome, they are considered ideal markers in many

diverse areas of research including the evolutionary history of primate populations (Brumfield et al., 2003). Compared with microsatellites or short tandem repeats (STRs), SNP loci are generally biallelic and therefore show lower genetic variation when few loci are used. However, with more SNP loci, the cumulative level of genetic variation can rival that of STRs but being more straightforward, and as thus SNPs are becoming more critical in studies involving the comparisons of genomic diversities and histories of different species (Brumfield et al., 2003). Recently, SNPs markers have been developed among macaque species, especially in *M. mulatta* (Satkoski et al., 2008b; Malhi et al., 2007; Street et al., 2007) which can be used for regional specific identification; Chinese or Indian (Ferguson et al., 2007). Genetic comparison of *M. mulatta* and *M. fascicularis* revealed highly conserved SNPs within these two species (Malhi et al., 2007). Moreover, previous study has designed SNPs panels (Ancestry and admixture) which were successful to estimate the level of genetic admixture between *M. mulatta* and *M. fascicularis* and the genetic differentiation within *M. fascicularis* populations (Zhang et al., 2017).

7. Phylogenetic tree analysis

Phylogenetic tree or phylogeny is a branch of diagram which shows the relationship between interested taxa (Baum, 2008). Basically, it is used to infer the evolutionary relationship among organisms based on the differentiation in their characteristics such as morphological, behavioral and genetic. The tip of the tree refers to each taxa and the node represents common ancestor between taxa as shown in Figure 2.18. The taxon outside the group of interest or outgroup is generally combined with those of interested taxa to gain more clearly scenario in evolutionary relationship.

Phylogenetic tree construction is divided into two main methods that are distance matrix and character state. The distance matrix compares the dissimilarity of each pair of taxon and calculates the pairwise distance matrix. Because of its rapid calculation, this method is suitable for large data sets. The example of this method are

UPGMA (Sokal & Michener, 1958) and Neighbor-joining (NJ) (Saitou & Nei 1987). In contrast, character state methods which are maximum parsimony (MP) (Farris, 1970; Fitch,1971), maximum likelihood (ML) (Felsenstein, 1981) and Bayesian analysis (Huelsenbeck & Ronquist, 2001) calculate the differentiation from each position (character) in any interested traits. For example; in any DNA alignment, each nucleotide in the same position (column) refers to each character, and all characters will be analyzed independently.

The reliability in a given phylogenetic tree can be assessed by two main computational methods that are bootstraps ranging from 0-100 (for NJ and ML) and posterior probability ranging from 0-1 (for Bayesian analysis). Thus, the higher value of each bootstraps and posterior probability indicates higher reliability of the phylogenetic result.

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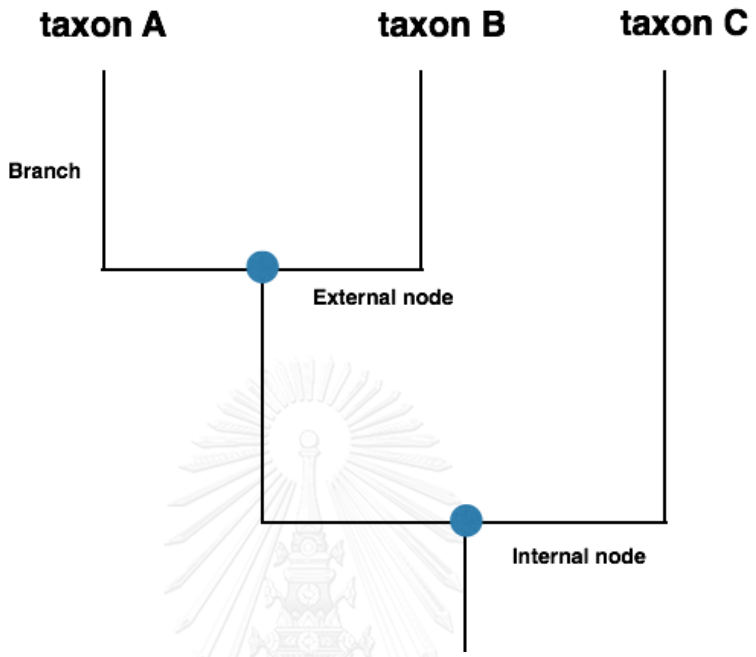

Figure 2. 18 Diagram of phylogenetic tree. The tip of phylogenetic tree refers to each taxon. Node refers to the common ancestor between two branches.

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8. STRUCTURE analysis

STRUCTURE program is one of the most popular softwares using to determine the population structure based on the multi-locus genotype data (Pritchard, Stephens, & Donnelly, 2000). In the analysis, Bayesian clustering approach is used to define number of clusters or populations (K) in which each of K is characterized by a set of alleles frequency at each locus. All individuals (samples) will be assigned (probabilistically) to a single population or join between two or more populations if they showed admixed genotypes (Pritchard, et al., 2000). Various types of genetic markers can be applied for this analysis such as STRs, SNPs and restriction fragment length polymorphisms (RFLPs). However, all markers are assumed to be in the condition of linkage equilibrium (all markers are unlinked) and Hardy-Weinberg's population (Pritchard et al., 2000). Four main models can be applied for the ancestry of individuals; (1) no admixture model which considers each population as a complete discrete; (2) admixture model which considers a mixed ancestry in individuals; (3) linkage model which considers the linked loci in admixed populations; (4) models with informative prior. The latest model allows the user to add a prior information about sampling locations that might relevant to the clustering, such as physical characteristics of sampled individuals or geographic sampling locations, in the analysis (Pritchard et al., 2000; Falush et al. 2003; Hubisz et al., 2009; Porras-Hurtado et al., 2013).

However, Evanno, Regnaut, & Goudet, (2005) suggested that K value obtained from STRUCTURE program does not correspond to the real number of the cluster while deltaK, which is estimated based on the second order derivation of the change in variance of the log probability between successive K values, is more accurate. Currently, STRUCTURE program is very useful for population genetics, for instance, to determine the evolutionary relationship of interested populations or to classify the unknown individual to a population.

CHAPTER III
MITOCHONDRIAL DNA AND TWO Y-CHROMOSOME GENES OF *Macaca fascicularis fascicularis* THROUGHOUT THAILAND AND VICINITY

Introduction

Macaca fascicularis have the second widest distribution range of all non-human primate species after *M. mulatta* (Wheatley et al., 1999). The distribution range of *M. fascicularis* covers a large part of Southeast Asia, including continental, peninsular and insular areas across Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia, Indonesia, the Philippines and Nicobar Islands which is a part of India (Fooden, 1995), although the former populations in Bangladesh are possibly extinct (Kabir & Ahsan, 2012). Along with their wide distribution and various habitat types, *M. fascicularis* shows regional differences in traits, such as pelage color, cheek hair pattern and tail length, and is classified into 10 subspecies of *M. f. fascicularis*, *M. f. aurea*, *M. f. phillipinensis*, *M. f. umbrosa*, *M. f. fusca*, *M. f. lasiae*, *M. f. atriceps*, *M. f. condorensis*, *M. f. tua* and *M. f. karimondjawae* (Fooden, 1995). Among those subspecies, *M. f. fascicularis* is the most well-known and commonly found throughout the species distribution range (Fooden, 1995, 2006; Groves, 2001).

The wide distribution of *M. f. fascicularis* spreads over the two biogeographical regions of Indochina and Sunda. The Indochinese region is a part of mainland Southeast Asia, including Myanmar, Vietnam, Laos, Cambodia and Thailand except for its southern peninsular part, while the Sundaic region comprises peninsular Thailand, Malaysia and Sunda (Sumatra, Java, Borneo, Bali and Lesser Sunda Islands until Timor Island). The landscape of this area has been repeatedly changed by glacial activities and sea level fluctuations (Lambeck & Chappell, 2001). In addition, climate changes, such as rainfall, have affected the vegetation of this area (Parnell, 2013), which has also

resulted in the current diverse distributions of animals. Thus, it is interesting to know what kind of biogeographic factors have affected the evolutionary history of *M. f. fascicularis* and formed their current distribution. To date, the phylogeography of *M. f. fascicularis* has been widely studied, but not fully understood. Although many genetic/genomic studies on *M. f. fascicularis* have been performed, most of them have focused on monkeys of unknown origin or known only to have originated in Malaysia, Indonesia or Vietnam (Stevison & Kohn, 2008; Blancher et al., 2008; Kanthaswamy et al., 2013). Only recently have studies focused on the phylogeography of *M. f. fascicularis* using sample of known detailed origins (Abdul-latiff et al., 2014b; Liedigk et al., 2015). Thus, phylogeographical studies of *M. f. fascicularis* with intensive sampling of many populations are awaited to uncover the evolutionary history of the species.

Within the distribution range of *M. f. fascicularis*, Thailand is likely a key location for investigation of their phylogeograhy because it connects the two distinct Indochina and Sunda regions and encompasses several biogeographic barriers. For instance, the high altitude Tanintharyi mountain range has hampered gene flow between two subspecies of *M. fascicularis* (*M. f. fascicularis* and *M. f. aurea*) (Bunlungsup et al., 2015). The Isthmus of Kra (approximately 10° 30´ N) and its marginal area, the most famous and the largest biogeographic barrier in the country, is known to be the range limit of several animal taxa, such as mammals (Corbett & Hill, 1992), reptiles (Inger & Voris, 2001) and forest birds (Hughes et al., 2003).

The Isthmus of Kra has also been proposed to have had strong effects on the phylogeography of *M. f. fascicularis*. Genetic studies have suggested that hybridization between *M. f. fascicularis* and *M. mulatta* occurred at the edge of their distribution ranges for over a million years (Osada et al., 2010). At present, their hybrid zone is located around 15–20° N across four countries (Myanmar, Thailand, Laos and Vietnam) (Fooden, 1964; Hamada et al., 2006). DNA sequences from the Y-chromosome of the northern *M. f. fascicularis* (Vietnam, Cambodia and Central Thailand) clustered with

those sequences of *M. mulatta*, but were separated from those of the southern *M. f. fascicularis* (Southern Thailand, Malaysia, Indonesia and the Philippines) (Tosi et al., 2002). On the other hand, mtDNA sequences of those *M. f. fascicularis* are monophyletic. Based on the Y-chromosome gene paraphyly, the authors proposed male mediated introgression from *M. mulatta* into *M. f. fascicularis*, and that the Klong Marui Fault at the center of the Isthmus of Kra was the limit of gene flow. However, two populations of *M. f. fascicularis* in Thailand that were included in the study (Tum Chompol and Songkla) were located quite far from the Isthmus of Kra (approximately 450 km and 400 km apart, respectively). Therefore, the exact location that separates the distribution of the two Y-chromosome clusters among *M. f. fascicularis*, which may represent the barrier for male mediated introgression from *M. mulatta*, is unknown.

To elucidate the evolutionary history of *M. f. fascicularis* and their hybridization with *M. mulatta*, the phylogeography of *M. f. fascicularis* populations throughout Thailand and the vicinity, where many biogeographic barriers are present, is the focal point of this study. In this study, genetic samples of *M. f. fascicularis* and *M. mulatta* were intensively collected and analyzed by means of DNA sequence analysis of a fragment of mtDNA and two Y-chromosome genes, using phylogenetic analysis. Since the social system of both *M. fascicularis* and *M. mulatta* is characterized as male dispersal and female philopatry, where males disperse from, but females remain within, their natal groups (Sade, 1972), the matrilineal mtDNA and patrilineal Y-chromosomal DNA markers were expected to show different aspects of *M. f. fascicularis* evolution.

Methods

Sample collection and species/subspecies identification

Blood and fecal samples were analyzed from 29 *M. f. fascicularis* and 5 *M. mulatta* populations throughout Thailand, including at their proposed hybrid zone (Fooden, 1964; Hamada et al., 2006), together with samples in Myanmar, China,

Vietnam and Indonesia (of those, five *M. f. fascicularis* and one *M. mulatta* populations had been studied in Bunlungsup et al. (2015), and its genetic data were retrieved and combined) (Table 3.1). All DNA samples were derived from DNA Bank of the Primate Research Unit, Chulalongkorn University which were collected in the past field survey (Malaivijitnond & Hamada, 2008; Malavijitnond et al., 2008). The species/subspecies status of the monkeys in each population was identified based on their morphological characters (Fooden 2006; Hamada et al., 2016) and previously reported distribution ranges, where *M. mulatta* occupies the northern part of the region at 15–36° N in the subtropical-temperate area, whereas *M. f. fascicularis* occupies the southern part at 21º N to 10º S in the tropical area (Fooden 1995, 2006) (Figure 3.1). The pelage color and RTL were used for identifying the species status; *M. mulatta* has a bipartite pelage color and a RTL of < 70%, while *M. fascicularis* has a longer RTL (> 90%) and no bipartite pelage pattern (Hamada et al., 2016). According to these characteristics, monkey populations in the proposed hybrid zone which showed mixed morphological characteristics were identified as 'either *M. mulatta* or *M. fascicularis*'. Since there are three subspecies of *M. fascicularis* that live in Thailand (*M. f. fascicularis*, *M. f. aurea* and *M. f. atriceps*) (Fooden, 1995), they were distinguished to the subspecies level but samples of *M. f. aurea* and *M. f. atriceps* were not included in this study. Subspecies *M. f. aurea* lives close to *M. f. fascicularis* in a small part of western/southern Thailand. These two subspecies were distinguished from each other by the difference in their cheek hair patterns, where *M. f. fascicularis* has a transzygomatic cheek hair pattern and *M. f. aurea* has an infrazygomatic pattern (Fooden, 1995; Bunlungsup et al., 2015). Subspecies *M. f. atriceps* is distributed in a small island of a limited area (Kho Kram Yai) in eastern Thailand (Fooden, 1995).

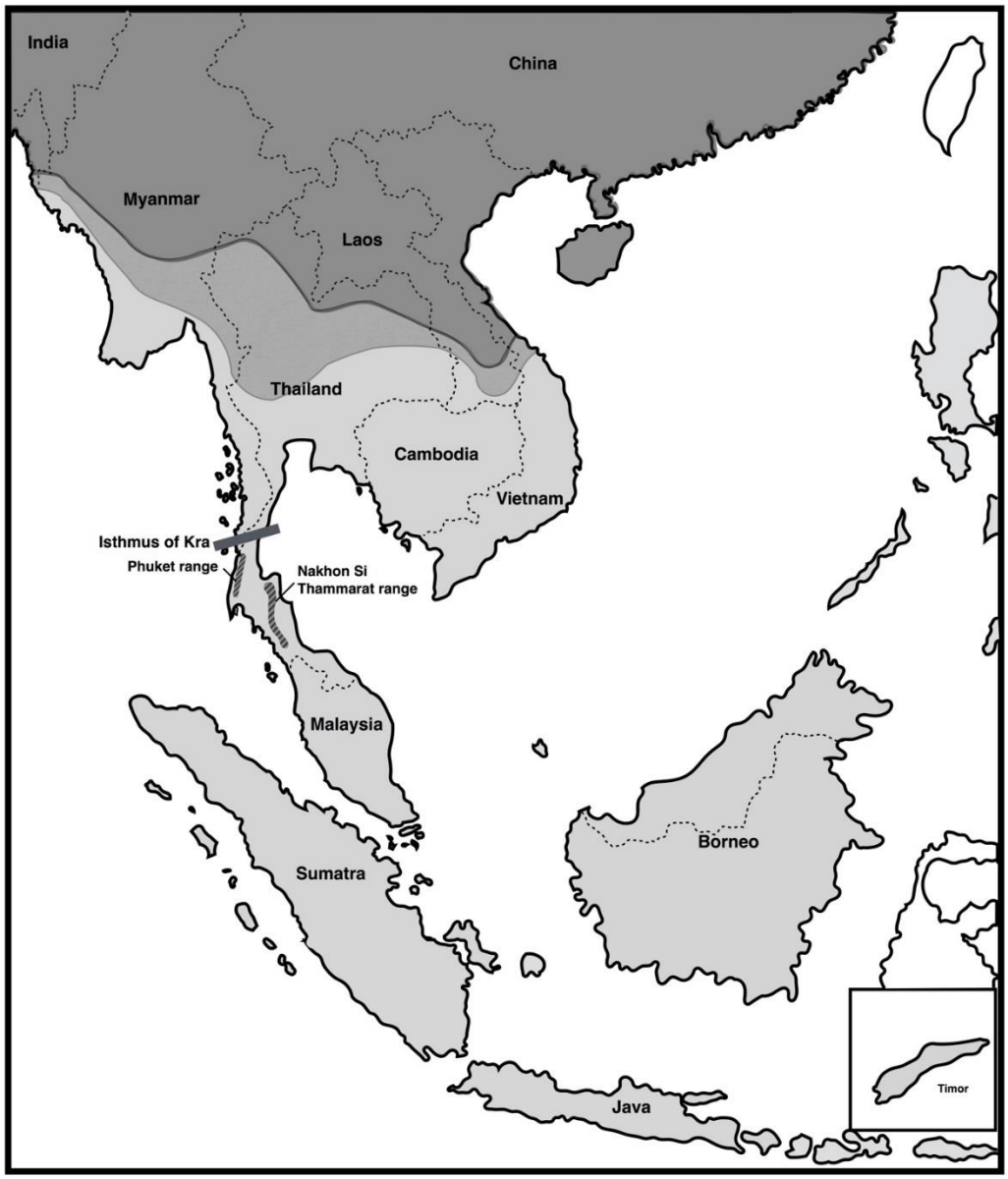

Figure 3.1 Distribution ranges of *M. mulatta* (dark grey), *M. fascicularis* (light grey) and their hybrid zone (grey).

Table 3. 1 Sample type, region, locality, geographical coordinate and genetic markers of the *M. mulatta* (*Mm*) and *M. f. fascicularis* (*Mff*) samples that were analyzed in this study.

Taxon	Sample type	Region	Name of location	GPS	mtDNA	*SRY-TPSY*
Mm	Blood	China	1. Reared at the Primate Research Institute of Kyoto University, Japan	*unknown*	Bunlungsup et al., 2015	Bunlungsup et al., 2015
	Blood	Myanmar	2. Shin ma Taung (SMT)	21° 58' N, 95° 10' E	This study	This study
	Blood	Thailand	3. Bann Sang School (BSS)	17° 51' N, 103° 57' E	This study	This study
	Blood		4. Wat Phattanajit (WPT)	17° 26' N, 104° 34' E	This study	This study
	Blood		5. Wat Tham Pa Mak Ho (WTPMH)	17° 14´ N, 101° 46´ E	This study	This study
Mff	Blood	(Indochina) Thailand	6. Wat Haad Moon (WHM)	16° 51´ N, 100° 28´ E	Bunlungsup et al., 2015	Bunlungsup et al., 2015
	Blood		7. Kosumphi Forest Park (KSP)	16° 15´ N, 103° 04´ E	This study	This study
	Blood		8. Wat Khao Nor (KN)	15° 57´ N, 99° 52´ E	Bunlungsup et al., 2015	Bunlungsup et al., 2015
	Blood		9. Wat Tham Thepbandan (WTT)	15° 44´ N, 101° 02´ E	This study	This study
	Feces		10. Wat Pikul Ngam (WPK)	15°16´ N, 100° 03´ E	This study	-
	Blood		11. Sai Yok (SY)	14° 07´ N, 99° 09´ E	This study	This study
	Blood		12. Wat Thammasala (WTM)	13° 48´ N, 100° 06´ E	This study	This study
	Blood		13. Khao Ngu Rock Garden (KNG)	13° 34´ N, 99° 46´E	This study	This study
	Feces		14. Wat Tham Khao Chaangon (WKCA)	13° 11´ N, 101° 34´ E	This study	-
	Feces		15. Tham Khao Ha Yod (TKH)	13° 9´ N, 101° 35´ E	This study	-
	Feces		16. Khao Tham Mee (KTM)	13° 8´ N, 101° 35´ E	This study	This study
	Blood		17. Wat Khao Thamon (WKT)	13° 02´ N, 99° 57´ E	Bunlungsup et al., 2015	This study
	Feces		18. Sai Keaw Beach (SKB)	12° 44´ N, 100° 50´ E	This study	This study
	Blood	Vietnam	19. Ca Mau Conservation (CMC)	Vietnam	This study	This study
	Blood	(Sundaic Thai gulf) Thailand	20. Suan Somdet Prasrinakharin Chumphon (SSD)	9° 56´ N, 99° 02´ E	This study	This study
	Feces		21. Tam Khao Keeree Wong (TKW)	9° 12´ N, 99° 39´ E	This study	-
	Feces		22. Wat Khao Keaw Wichian (WKK)	8° 12´ N, 100° 05´ E	This study	-
	Feces		23. Wat Khuha Sawan (WKS)	7° 37´ N, 100° 04´ E	This study	-
	Blood		24. Khao Chai Son (KCS)	7° 27´ N, 100° 07´ E	This study	-
	Blood		25. Khao Noi/Khao Tangkuan (KN/KTK)	7° 12´ N, 100° 35´ E	Bunlungsup et al., 2015	Bunlungsup et al., 2015
	Blood		26. Wat Khuha Phimuk (WKH)	6° 31´ N, 101° 13´ E	This study	This study
	Feces	(Sundaic Andaman Sea) Thailand	27. Suan Somdet Prasrinakharin Phangnga (SSP)	8° 25´ N, 98° 31´ E	This study	-
	Blood		28. Wat Suwankhuha (WSK)	8° 25´ N, 98° 28´ E	Bunlungsup et al., 2015	Bunlungsup et al., 2015
	Feces		29. Panak Island (PNI)	8° 12´ N, 98° 29´ E	This study	-
	Feces		30. Wat Tham Sue (WTS)	8° 07´ N, 98° 55´ E	This study	-
	Feces		31. Lanta Island (LTI)	7° 28´ N, 99° 05´ E	This study	-
	Feces		32. Khao Toh Phyawang (KTP)	6° 37´ N, 100° 03´ E	This study	This study
	Blood	Indonesia	33. Sibanganding (SBG)	2° 41´ N, 98° 55´ E	This study	-
	Blood		34. Barastagi (BRT)	3° 12´ N, 98° 32´ E	This study	This study

Blood samples were collected from temporarily caught monkeys as previously described by Malaivijitnond et al. (2008). After collection, blood was centrifuged at 1000g for 10 min and the buffy coat, which contains white blood cells, was harvested for DNA extraction. Genomic DNA was extracted using the standard phenol-chloroform method (Sambrook, Fritsch, & Maniatis, 1989).

Rectal cells on the surface of fecal samples of free-ranging macaques were collected in 1.7 ml of lysis buffer (0.5% (w/v) SDS, 100 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0 and 10 mM NaCl) (Hayaishi & Kawamoto, 2006). In brief, the surface of the fecal sample was swabbed with a cotton bud soaked in lysis buffer and then the cotton bud was dipped and stirred into the lysis buffer in a collection tube. This procedure was repeated at least three times per fecal sample in order to maximize the number of rectal cells and hence DNA yield. Samples were kept at room temperature before DNA extraction was performed using a QIAamp DNA stool mini kit (QIAGEN Inc., Hilden, Germany) following the manufacturer's protocol. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Faculty of Science in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University, Thailand, Protocol Review no. 1423010.

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PCR and sequencing

An approximately 835 bp fragment of mtDNA, including the hypervariable region I (HVSI), tRNA proline, tRNA threonine and part of the cytochrome b gene, was PCR amplified using the primers HVS-F (5´-CCGCCCACTCAGCCAATTCCTGTTC T-3´) and HVS-R (5´-CCCGTGATCCATCGAGATGTCTT-3´) (Smith & McDonough, 2005), and the amplicon sequence was determined. The primer pair was designed to avoid amplifying nuclear insertions of mtDNA (Smith & McDonough, 2005). However, to ensure that no nuclear insertion was amplified in this study, some samples were randomly picked and performed the DNA cloning. The results showed 100% homology between direct and cloned sequences. For the Y-chromosome, the sex-determining

region Y (*SRY*) and a part of the testis-specific protein Y (*TSPY*), which were approximately 800 bp and 2.3 kbp in length, respectively, were PCR amplified and the sequences were determined. The *SRY* gene was amplified using the primers SW2 (5´CTTGAGAATACATTGTCAGGG-3´) and SW3B (5´AGGTCTTTG TAGCCAAT GTTAC-CCG-3´) (Whitfield, Lovell-Badge, & Goodfellow, 1993), while the *TSPY* gene was amplified using the primers TSPY-A (5´-AGCCAGGAAGGCCTTTTCTCG-3´) and TSPY-5R (5´-CTGTGCATAAGACCATGCTGAG-3´) (Tosi et al., 2000).

All PCR reactions were performed in a 10 µl final volume containing 0.625 U of Hotstart ExTaq DNA Polymerase (Takara Bio Inc., Shiga, Japan), 0.2 µM of each primer and an adequate amount of genomic DNA template in the manufacturer's buffer plus 0.12 µg of T4 gene 32 protein (Wako Nippon Gene, Japan) to promote DNA synthesis. The PCR thermal cycling of the mtDNA fragment was performed at 94° C for 1 min, followed by 40 cycles at 94° C for 30 s, 60–63° C (-0.1° C/cycle) for 30 s and 72° C for 20 s, and then a final 72° C for 7 min. The conditions for *SRY* amplification were as above except the annealing stage was 52–57° C for 20 s. The PCR cycling conditions for *TSPY* amplification were 94° C for 1 min, followed by 40 cycles at 94° C for 30 s, 66° C for 45 s and 72° C for 3 min, and then a final 72° C for 7 min. The fecal samples which failed to amplify the 2.3 kbp *TSPY* fragment, perhaps because of template DNA fragmentation or a low quality of the template DNA, were amplified using 3 primer pairs (TSPYA-TSR1012, TSF566-TSR1676 and TDF1383-TSPY5R) as shown in Table 3.2 and Figure 3.2. All PCR products were separated by 1% (w/v) agarose-TAE gel electrophoresis and visualized by UV transillumination. When multiple bands were detected the target PCR product was selected based on size, cut out and extracted using a Fast Gene Gel/PCR Extraction Kit (Nippon Genetics Co., Japan). Otherwise the PCR amplicons were purified by ethanol precipitation or by using an ExoSAP-IT kit (Affymetrix Inc., CA, USA).

Sequencing was performed for both strands. For the mtDNA and *SRY* gene, the primers used for PCR were used for sequencing. For *TSPY*, in addition to the PCR primers, two internal primer pairs were used as shown in Table 3.2 and Figure 3.2. These internal primers could provide the partially overlapping region of sequences which was useful for sequence assembly. Sequencing was performed with an ABI 3130*xL* Genetic Analyzer (Applied Biosystems, CA, USA) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). Sequencing data were assembled and analyzed using SeqMan™ II (DNASTAR Inc., USA) or Finch TV (Geospiza Inc., USA).

It was reported recently that the TSPY gene had at least five forms of polymorphism in Mm, TSPY1, TSPY2, TSPY3, TSPY4, and TSPY5 (Hughes et al., 2012). To ensure that the PCR amplification and direct sequencing of the TSPY gene of macaques in our study were taken from the same form, the PCR amplicons of some monkeys were randomly selected and cloned. Three pairs of the primers of TSPY gene (Table 3.2) were used for sequencing. The 100% homology of sequences was shown between the direct and cloned sequences.

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Table 3. 2 Primer and annealing temperature for each marker using in this study

Primer	Nucleotide (5´ to 3´)	Annealing temperature
HVS-F	CCG-CCC-ACT-CAG-CCA-ATT-CCT-GTT-CT	60-63°C
HVS-R	CCC-GTG-ATC-CAT-CGA-GAT-GTC-TT	
SW2	CTT-GAG-AAT-ACA-TTG-TCA-GGG	52-57°C
SW3B	AGG-TCT-TTG-TAG-CCA-ATG-TTA-CCC-G	
TSPY-A	AGC-CAG-GAA-GGC-CTT-TTC-TCG	66-68°C
TSR1012	TGT-CAC-CTG-TGA-CGT-TCA-CGA	
TSF566	AGG-TCA-TTC-ATG-GAT-GCA-GAT	62-65°C
TSR1676	CCA-CAG-TTA-TAA-CCT-GCT-TTG-C	
TSF1383	AAT-CCC-CTG-CAA-TAC-TAC-AGG-AGG	62-66°C
TSPY-5R	CTG-TGC-ATA-AGA-CCA-TGC-TGA-G	

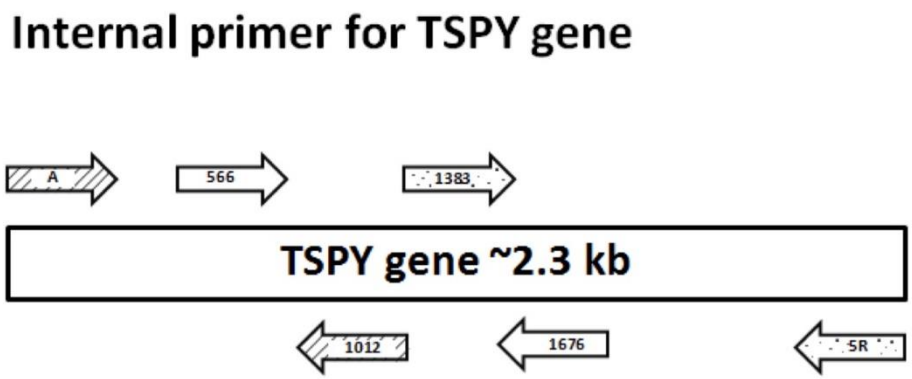

Figure 3. 2 Internal primers used in this study for the PCR amplification of the TSPY gene. A, 566, and 1383 strand for forward primers of TSPY-A, TSF566, and TSF1383, respectively. 5R, 1676, and 1012 strand for reverse primers of TSPY-5R, TSR1676, and TSR1012, respectivel

Data and phylogenetic tree analyses

Although the populations sampled in this study covered most of the distribution range of *M. f. fascicularis* in mainland Southeast Asia, the sampled populations from insular Southeast Asia were limited to a part of Sumatra. Therefore, to uncover a comprehensive scenario of *M. f. fascicularis* evolution, the additional sequences of *M. f. fascicularis* with known origins obtained from the GenBank database for further analyses were included.

For mtDNA, 33 sequences from individuals that were known to have originated in Thailand (n = 2), west Malaysia (n = 5), north Sumatra (n = 6), Bangka (n = 2), Java (n = 5), Borneo (n = 5) and Timor (n = 8) were included (Liedigk et al., 2015) (Table 3.3), which resulted in a final mtDNA data set of 129 sequences (114 and 15 sequences of *M. f. fascicularis* and *M. mulatta*, respectively).

For the Y-chromosome, 17 sequences of both the *SRY* and *TSPY* genes from samples that originated in Thailand (n = 2), Cambodia (n = 2), Vietnam (n = 2), west Malaysia (n = 3), east Malaysia (n = 2), Sumatra (n = 4), Java (n = 1) and Borneo (n = 1) were included (Tosi et al., 2002; Tosi, Morales, & Melnick, 2003) (Table 3.3), which resulted in two data sets (*SRY* and *TSPY*) each comprised of 65 sequences (54 and 11 sequences for *M. fascicularis* and *M. mulatta*, respectively). However, since no conflict was found between the *SRY* and *TSPY* sequences when the partition homogeneity was tested on PAUP 4.0 (Swofford, 2003) with 1,000 bootstraps (p = 1.0), these two sequence data sets were combined into a single concatenated *SRY-TSPY* data set. For phylogenetic tree construction, the sequences of *M. sylvanus* obtained from the GenBank database were used as an outgroup reference for both the mtDNA and *SRY-TSPY* trees (Accession numbers: NC_002764 for mtDNA, AF284326 for *SRY* and AF284275 for *TSPY*).

Phylogenetic analysis of both the mtDNA and *SRY-TSPY* sequences were performed using Bayesian analysis and maximum likelihood (ML) methods. For the Bayesian analysis, the best substitution model was selected under the Bayesian

information criterion (BIC) using Mega 5.2 (Tamura et al., 2011); where the HKY+I+G model was selected for the mtDNA data set and the JC model for the *SRY-TSPY* dataset. Bayesian analysis was conducted based on Malkov Chain Monte Carlo (MCMC) algorithm using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Two independent runs were carried out for 10,000,000 generations and parameters were collected every 1,000 generations. By using Tracer v.1.5 (Rambaut & Drummond, 2009), the convergence of each parameter between the two runs was confirmed and that effective sample size (ESS) exceeded 200 for all parameters. The first 25% of data (2,500 trees) for each run were discarded as burn-in, the remaining data were combined and a 50% majority rule consensus tree with posterior probability on each branch was summarized. The ML analysis was performed using RAxML8.2.0 (Stamatakis, 2014) with the GTRgamma model and 1,000 rapid bootstrap replications for both the mtDNA and *SRY-TSPY* data sets. Optimal trees from MrBayes and RAxML were visualized by FigTree v.1.4.2. (Rambaut, 2014).

Divergence time estimation

Divergence time of mtDNA was estimated by Bayesian framework using BEAST 2.4.2 (Bouckaert et al., 2014). For substitution model, HKY+I+G model was used as same as for the Bayesian phylogenetic inference described above. Uncorrelated Relaxed Log Normal Clock model and Coalescence Bayesian Skyline tree prior were selected. For time calibration, the divergence of African and Asian macaque lineages at 5.5 million years ago (MYA) (4.5-6.5 MYA: 95% Credibility Interval (CI)) was used (Alba et al., 2014) assuming normal distribution prior. Two independent runs with 50,000,000 generations were run and parameters were collected every 1,000 generations. Convergence and ESS were checked by Tracer as same as in the phylogenetic tree inference. After discarding the first 25% of the data, sampled parameters of the two runs were combined using LogCombiner and a consensus tree with estimated divergence

time was generated using TreeAnnotator. The generated tree was visualized by FigTree v.1.4.2.

Table 3. 3 Detailed information of the region, name and accession number of downloaded sequences from the GenBank database which were combined with this analysis.

Region	Name	Accession number		
		mtDNA	***SRY***	***TSPY***
Thailand	1. MFAS4	KM851024	-	-
	2. MFAS5	KM851036	-	-
W. Malaysia	3 MFAS6	KM851016	-	-
	4. MFAS7	KM851012	-	-
	5. MFAS8	KM851018	-	-
	6. MFAS9	KM851017	-	-
	7. MFAS10	KM851015	-	-
N. Sumatra	8. MFAS11	KM850999	-	-
	9. MFAS12	KM851010	-	-
	10. MFAS13	KM851011	-	-
	11. MFAS14	KM850998	-	-
	12. MFAS15	KM851022	-	-
	13. MFAS16	KM851035	-	-
Bangka	14. MFAS17	KM851023	-	-
	15. MFAS18	KM851034	-	-
Java	16. MFAS19	KM851031	-	-
	17. MFAS20	KM851021	-	-
	18. MFAS21	KM851029	-	-
	19. MFAS22	KM851020	-	-
	20. MFAS23	KM851028	-	-
Timor	21. MFAS24	KM851033	-	-
	22. MFAS25	KM851026	-	-
	23. MFAS26	KM851025	-	-
	24. MFAS27	KM851019	-	-
	25. MFAS28	KM851027	-	-
	26. MFAS29	KM851037	-	-
	27. MFAS30	KM851030	-	-
	28. MFAS31	KM851032	-	-
Borneo	29. MFAS32	KM851014	-	-
	30. MFAS38	KM851002	-	-
	31. MFAS39	KM851008	-	-
	32. MFAS40	KM851006	-	-
	33. MFAS41	KM851013	-	-
Cambodia	34. Camb3	-	AF425286	AF425271
	35. Camb4	-	AF425287	AF425272
Thailand	36. Tum_chompol	-	AF425288	AF425273
	37. Songkla	-	AF425295	AF425280
Vietnam	38. Vietnam290WB	-	AF284304	AF284253
	39. VietnamSV4	-	AF425284	AF425269

Region	Name	Accession number		
		mtDNA	***SRY***	***TSPY***
W. Malaysia	40. Johor_DJ95	-	AF425292	AF425277
	41. Selangor_UKM003	-	AF425293	AF425278
	42. Selangor_UKM004	-	AF425294	AF425279
E. Malaysia	43. Sepilok	-	AF284300	AF284249
	44. Sarawak	-	AF284299	AF284248
S. Sumatra	45. S_Sumatra A12133	-	DQ832603	DQ832617
	46. S_Sumatra A9652	-	DQ832602	DQ832616
	47. S_Sumatra A8097	-	DQ832601	DQ832615
	48. S_Sumatra A1828	-	DQ832600	DQ832614
Java	49. Java_34	-	AF284303	AF284252
Borneo	50. Borneo PM666	-	AF284302	AF284251
Outgroup	*Macaca sylvanus*	NC_002764	AF284326	AF284275

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Results

Nucleotide sequences of *M. f. fascicularis* and *M. mulatta*

For the mtDNA data, 63 and 12 sequences of *M. f. fascicularis* and *M. mulatta,* of 679 bp in length were deposited in GenBank database (Accession numbers: LC166970-LC167044) (Table 3.4). Based on the final data, which included the downloaded sequences (Table 3.3), 182 variable sites (26.8%) were found of which 174 sites were parsimony informative. The number of transitions and transversions for two variants was 153 and seven, respectively, giving an approximately 22:1 transition/transversion ratio. However, there were in addition three and four transition and transversion variants. They were counted as both types, causing a total number of transitions and transversions was 167 and 21, respectively (approximately 8:1 transition/transversion ratio).

For the *SRY-TSPY* data, 25 sequences of both the *SRY* and *TSPY* gene fragments for *M. f. fascicularis* and likewise 10 for *M. mulatta* were deposited in the GenBank database (Accession numbers: LC167102-LC167136 for *SRY* and LC167045-LC167079 for *TSPY*) (Table 3.4). Based on the final data, which included the downloaded sequences (Table 3.3), only 10 variable sites were found among the 2,726 bp of the concatenated *SRY-TSPY* gene sequences, of which nine sites were parsimony informative and were comprised of four transitions and five transversions, giving a 0.8:1 transition/transversion ratio. There was a one-bp indel in *TSPY* and a three-bp indel in *SRY*.

Table 3.4 Accession numbers of new samples which were analyzed in this study. *Mff* and *Mm* stand for *M. f. fascicularis* and *M. mulatta*, respectively

Genetic Marker	Accession number	No.	Organism	Original /isolate	Country
mtDNA	LC167007	A-38)	*Mff*	TKH2126	Thailand:Chon buri, Tham Khao Ha Yod
	LC167008	A-39)	*Mff*	TKH2127	Thailand:Chon buri, Tham Khao Ha Yod
	LC167009	A-40)	*Mff*	TKW1836	Thailand:Surat Thani, Tam Khao Keeree Wong
	LC167010	A-41)	*Mff*	TKW1837	Thailand:Surat Thani, Tam Khao Keeree Wong
	LC167011	A-42)	*Mff*	WKCA2120	Thailand:Chon buri, Wai Tham Khao Chaangon
	LC167012	A-43)	*Mff*	WKCA2123	Thailand:Chon buri, Wai Tham Khao Chaangon
	LC167013	A-44)	*Mff*	WKCA2124	Thailand:Chon buri, Wai Tham Khao Chaangon
	LC167014	A-45)	*Mff*	WKH274	Thailand:Yala, Wat Khuha Phimuk
	LC167015	A-46)	*Mff*	WKH276	Thailand:Yala, Wat Khuha Phimuk
	LC167016	A-47)	*Mff*	WKH277	Thailand:Yala, Wat Khuha Phimuk
	LC167017	A-48)	*Mff*	WKK1895	Thailand:Nakhon Si Thammarat, Wat Khao Keaw Wichian
	LC167018	A-49)	*Mff*	WKK1896	Thailand:Nakhon Si Thammarat, Wat Khao Keaw Wichian
	LC167019	A-50)	*Mff*	WKS1897	Thailand:Phatthalung, Wat Khuha Sawan
	LC167020	A-51)	*Mff*	WKS1898	Thailand:Phatthalung, Wat Khuha Sawan
	LC167021	A-52)	*Mff*	WKS1901	Thailand:Phatthalung, Wat Khuha Sawan
	LC167022	A-53)	*Mff*	WPK2020	Thailand:Chai Nat, Wat Pikul ngam
	LC167023	A-54)	*Mff*	WPK2021	Thailand:Chai Nat, Wat Pikul ngam
	LC167024	A-55)	*Mff*	WPK2023	Thailand:Chai Nat, Wat Pikul ngam
	LC167025	A-56)	*Mff*	WTM709	Thailand:Nakhon Pathom, Wat Thammasala
	LC167026	A-57)	*Mff*	WTM710	Thailand:Nakhon Pathom, Wat Thammasala
	LC167027	A-58)	*Mff*	WTM711	Thailand:Nakhon Pathom, Wat Thammasala
	LC167028	A-59)	*Mff*	WTM712	Thailand:Nakhon Pathom, Wat Thammasala
	LC167029	A-60)	*Mff*	WTS1986	Thailand:Krabi, Wat Tham Sue
	LC167030	A-61)	*Mff*	WTS1988	Thailand:Krabi, Wat Tham Sue
	LC167031	A-62)	*Mff*	WTT850	Thailand:Phetchabun, Wat Tham Thepbandan
	LC167032	A-63)	*Mff*	WTT853	Thailand:Phetchabun, Wat Tham Thepbandan
	LC167033	A-64)	*Mm*	BSS983	Thailand:Nong Khai, Bann Sang School
	LC167034	A-65)	*Mm*	BSS985	Thailand:Nong Khai, Bann Sang School
	LC167035	A-66)	*Mm*	BSS986	Thailand:Nong Khai, Bann Sang School
	LC167036	A-67)	*Mm*	BSS987	Thailand:Nong Khai, Bann Sang School
	LC167037	A-68)	*Mm*	SMT1457	Myanmar:Shin Ma Tang
	LC167038	A-69)	*Mm*	SMT1458	Myanmar:Shin Ma Tang
	LC167039	A-70)	*Mm*	SMT1459	Myanmar:Shin Ma Tang
	LC167040	A-71)	*Mm*	WPT1047	Thailand:Nakhon Phanom, Wat Phattanajit
	LC167041	A-72)	*Mm*	WPT1048	Thailand:Nakhon Phanom, Wat Phattanajit
	LC167042	A-73)	*Mm*	WPT1049	Thailand:Nakhon Phanom, Wat Phattanajit
	LC167043	A-74)	*Mm*	WTPMH242	Thailand:Loei, Wat Tham Pa Mak Ho
	LC167044	A-75)	*Mm*	WTPMH247	Thailand:Loei, Wat Tham Pa Mak Ho
TSPY	LC167045	B-1)	*Mff*	Berastagi3	Indonesia:Berastagi
	LC167046	B-2)	*Mff*	Berastagi4	Indonesia:Berastagi
	LC167047	B-3)	*Mff*	CMC1170	Viet Nam:Ca Mau

Genetic Marker	Accession number	No.	Organism	Original /isolate	Country
TSPY	LC167048	B-4)	*Mff*	CMC1174	Viet Nam:Ca Mau
	LC167049	B-5)	*Mff*	KNG329	Thailand:Ratchaburi, Khao Ngu Rock Garden
	LC167050	B-6)	*Mff*	KNG330	Thailand:Ratchaburi, Khao Ngu Rock Garden
	LC167051	B-7)	*Mff*	KNG331	Thailand:Ratchaburi, Khao Ngu Rock Garden
	LC167052	B-8)	*Mff*	KSP142	Thailand:Maha Sarakham, Kosumphi Forest Park
	LC167053	B-9)	*Mff*	KSP143	Thailand:Maha Sarakham, Kosumphi Forest Park
	LC167054	B-10)	*Mff*	KSP144	Thailand:Maha Sarakham, Kosumphi Forest Park
	LC167055	B-11)	*Mff*	KSP145	Thailand:Maha Sarakham, Kosumphi Forest Park
	LC167056	B-12)	*Mff*	KTM2135	Thailand:Chon buri, Khao Tham Mee
	LC167057	B-13)	*Mff*	KTP1903	Thailand:Satun, Khao Toh Phyawang
	LC167058	B-14)	*Mff*	SKB2146	Thailand:Chon buri, Sai Keaw Beach
	LC167059	B-15)	*Mff*	SSD1200	Thailand:Chumphon, Suan Somdet Prasrinakharin Chumphon
	LC167060	B-16)	*Mff*	SSD1208	Thailand:Chumphon, Suan Somdet Prasrinakharin Chumphon
	LC167061	B-17)	*Mff*	SY1162	Thailand:Kanchanaburi, Sai Yok
	LC167062	B-18)	*Mff*	WKH277	Thailand:Yala, Wat Khuha Phimuk
	LC167063	B-19)	*Mff*	WKH282	Thailand:Yala, Wat Khuha Phimuk
	LC167064	B-20)	*Mff*	WSK883	Thailand:Phang nga, Wat SuwanKhuha
	LC167065	B-21)	*Mff*	WTM709	Thailand:Nakhon Pathom, Wat Thammasala
	LC167066	B-22)	*Mff*	WTM710	Thailand:Nakhon Pathom, Wat Thammasala
	LC167067	B-23)	*Mff*	WTM712	Thailand:Nakhon Pathom, Wat Thammasala
	LC167068	B-24)	*Mff*	WTM713	Thailand:Nakhon Pathom, Wat Thammasala
	LC167069	B-25)	*Mff*	WTT849	Thailand:Phetchabun, Wat Tham Thepbandan
	LC167070	B-26)	*Mm*	BSS983	Thailand:Nong Khai, Bann Sang School
	LC167071	B-27)	*Mm*	BSS986	Thailand:Nong Khai, Bann Sang School
	LC167072	B-28)	*Mm*	China570	China
	LC167073	B-29)	*Mm*	SMT1458	Myanmar:Shin Ma Tang
	LC167074	B-30)	*Mm*	SMT1459	Myanmar:Shin Ma Tang
	LC167075	B-31)	*Mm*	WPT1047	Thailand:Nakhon Phanom, Wat Phattanajit
	LC167076	B-32)	*Mm*	WPT1050	Thailand:Nakhon Phanom, Wat Phattanajit
	LC167077	B-33)	*Mm*	WTPMH242	Thailand:Loei, Wat Tham Pa Mak Ho
	LC167078	B-34)	*Mm*	WTPMH244	Thailand:Loei, Wat Tham Pa Mak Ho
	LC167079	B-35)	*Mm*	WTPMH247	Thailand:Loei, Wat Tham Pa Mak Ho
	LC167102	C-1)	*Mff*	Berastagi3	Indonesia:Berastagi
SRY	LC167103	C-2)	*Mff*	Berastagi4	Indonesia:Berastagi
	LC167104	C-3)	*Mff*	CMC1170	Viet Nam:Ca Mau
	LC167105	C-4)	*Mff*	CMC1174	Viet Nam:Ca Mau
	LC167106	C-5)	*Mff*	KNG329	Thailand:Ratchaburi, Khao Ngu Rock Garden
	LC167107	C-6)	*Mff*	KNG330	Thailand:Ratchaburi, Khao Ngu Rock Garden
	LC167108	C-7)	*Mff*	KNG331	Thailand:Ratchaburi, Khao Ngu Rock Garden
	LC167109	C-8)	*Mff*	KSP142	Thailand:Maha Sarakham, Kosumphi Forest Park
	LC167110	C-9)	*Mff*	KSP143	Thailand:Maha Sarakham, Kosumphi Forest Park

Genetic Marker	Accession number	No.	Organism	Original /isolate	Country
SRY	LC167111	C-10)	*Mff*	KSP144	Thailand:Maha Sarakham, Kosumphi Forest Park
	LC167112	C-11)	*Mff*	KSP145	Thailand:Maha Sarakham, Kosumphi Forest Park
	LC167113	C-12)	*Mff*	KTM2135	Thailand:Chon buri, Khao Tham Mee
	LC167114	C-13)	*Mff*	KTP1903	Thailand:Satun, Khao Toh Phyawang
	LC167115	C-14)	*Mff*	SKB2146	Thailand:Chon buri, Sai Keaw Beach
	LC167116	C-15)	*Mff*	SSD1200	Thailand:Chumphon, Suan Somdet Prasrinakharin Chumphon
	LC167117	C-16)	*Mff*	SSD1208	Thailand:Chumphon, Suan Somdet Prasrinakharin Chumphon
	LC167118	C-17)	*Mff*	SY1162	Thailand:Kanchanaburi, Sai Yok
	LC167119	C-18)	*Mff*	WKH277	Thailand:Yala, Wat Khuha Phimuk
	LC167120	C-19)	*Mff*	WKH282	Thailand:Yala, Wat Khuha Phimuk
	LC167121	C-20)	*Mff*	WSK883	Thailand:Phang nga, Wat SuwanKhuha
	LC167122	C-21)	*Mff*	WTM709	Thailand:Nakhon Pathom, Wat Thammasala
	LC167123	C-22)	*Mff*	WTM710	Thailand:Nakhon Pathom, Wat Thammasala
	LC167124	C-23)	*Mff*	WTM712	Thailand:Nakhon Pathom, Wat Thammasala
	LC167125	C-24)	*Mff*	WTM713	Thailand:Nakhon Pathom, Wat Thammasala
	LC167126	C-25)	*Mff*	WTT849	Thailand:Phetchabun, Wat Tham Thepbandan
	LC167127	C-26)	*Mm*	BSS983	Thailand:Nong Khai, Bann Sang School
	LC167128	C-27)	*Mm*	BSS986	Thailand:Nong Khai, Bann Sang School
	LC167129	C-28)	*Mm*	China570	China
	LC167130	C-29)	*Mm*	SMT1458	Myanmar:Shin Ma Tang
	LC167131	C-30)	*Mm*	SMT1459	Myanmar:Shin Ma Tang
	LC167132	C-31)	*Mm*	WPT1047	Thailand:Nakhon Phanom, Wat Phattanajit
	LC167133	C-32)	*Mm*	WPT1050	Thailand:Nakhon Phanom, Wat Phattanajit
	LC167134	C-33)	*Mm*	WTPMH242	Thailand:Loei, Wat Tham Pa Mak Ho
	LC167135	C-34)	*Mm*	WTPMH244	Thailand:Loei, Wat Tham Pa Mak Ho
	LC167136	C-35)	*Mm*	WTPMH247	Thailand:Loei, Wat Tham Pa Mak Ho

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Phylogenetic inference based on mtDNA

The mtDNA phylogenetic trees constructed by Bayesian and ML analysis showed a similar topology with the same relationships between each clade/subclade, and each tree result of Bayesian (Figure 3.4) and ML (Figure 3.5) were shown here. All macaques were divided into two main clades, *M. mulatta* and *M. f. fascicularis*, which accorded with the species identification except for one *M. mulatta* population (Wat Tham Pa Mak Ho; WTPMH, no.5 in Table 3.1 and Figure 3.3) that was grouped within the *M. f. fascicularis* clade. With respect to the high genetic variation in the HVSI region of the mtDNA together with female philopatry, *M. mulatta* and *M. f. fascicularis* were separated into many subclades in accord with their distribution. The clade of *M. mulatta* was divided into two subclades; one from China (China, no.1) and another from Myanmar (Shin Ma Thaung; SMT, no.2) and Thailand (Wat Phattanajit (WPT, no.4) and Ban Sang School (BSS, no.3)). Within the *M. f. fascicularis* clade, five subclades were recognized (Insular Indonesia, Sundaic Thai Gulf, Vietnam, Sundaic Andaman Sea Coast and Indochina). Among these subclades, the Insular Indonesian *M. f. fascicularis* (Timor, Borneo, Java and Bangka) had been separated first from the other subclades and then the Sundaic Thai Gulf subclade separated from the others. The Sundaic Thai Gulf subclade was comprised of only Thai macaque populations that ranged the Thai Gulf side (Wat Khuha Sawan (WKS, no.23), Khao Noi/Khao Tangkuan (KN/KTK, no.25), Wat Khao Keaw Wichian (WKK, no.22), Tam Khao Keeree Wong (TKW, no.21), Suan Somdet Prasrinakharin Chumphon (SSD, no.20) and Khao Chai Son (KCS, no.24)). Next, the Vietnam subclade, Ca Mau Conservation Area (CMC, no.19), was split from the remaining subclades and lastly the Sundaic Andaman Sea Coast and Indochina subclades diverged. The Sundaic Andaman Sea Coast subclade was comprised of both Thai macaques that occupied the Andaman Sea Coast side (Khao Toh Phyawang (KTP, no.32), Lanta Island (LTI, no.21), Wat Tham Sue (WTS, no.30) and Suan Somdet Prasrinakharin Phangnga (SSP, no.27)) and northern Sumatra macaques (Berastagi

(BRT, no.34) and Sibaganding (SBG, no.33)) along with the downloaded sequences from west Malaysia (MFAS6–MFAS10) and northern Sumatra (MFAS11–MFAS16). The Indochina subclade was comprised of all the Thai macaques that occupied the Indochina region (Wat Tham Thepbandan (WTT, no.9), Kosumphi Forest Park (KSP, no.7), Wat Pikul Ngam (WPK, no.10), Wat Tham Khao Cha-ang-on (WKCA, no.14), Tham Khao Har Yod (TKH, no.15), Khao Tham Mee (KTM, no.16), Sai Keaw Beach (SKB, no.18), Wat Thammasala (WTM, no.12), Wat Khao Thamon (WKT, no.17), Wat Haad Moon (WHM, no.6), Sai Yok (SY, no.11), Khao Ngu Rock Garden (KNG, no.13) and Wat Khao Nor (KN, no.8)) together with the downloaded sequences from Thailand (MFAS4–5) and WTPMH (a *M. mulatta* population based on their morphological characters). However, some *M. f. fascicularis* populations were not clustered according to their regional origins. Wat Khuha Phimuk (WKH, no. 26) was a *M. f. fascicularis* population living in the Thai gulf side but was grouped within the Andaman Sea Coast subclade. In contrast, Wat Suwankhuha (WSK, no. 28) and Panak Island (PNI, no. 29) located in the Andaman Sea Coast side were grouped within the Thai gulf subclade. Interestingly, these two populations (PNI and WSK) were distributed only in the narrow area of 8° 25´ to 8° 12´ N, 98° 28´ to 98° 29´ E in the southern part of Thailand.

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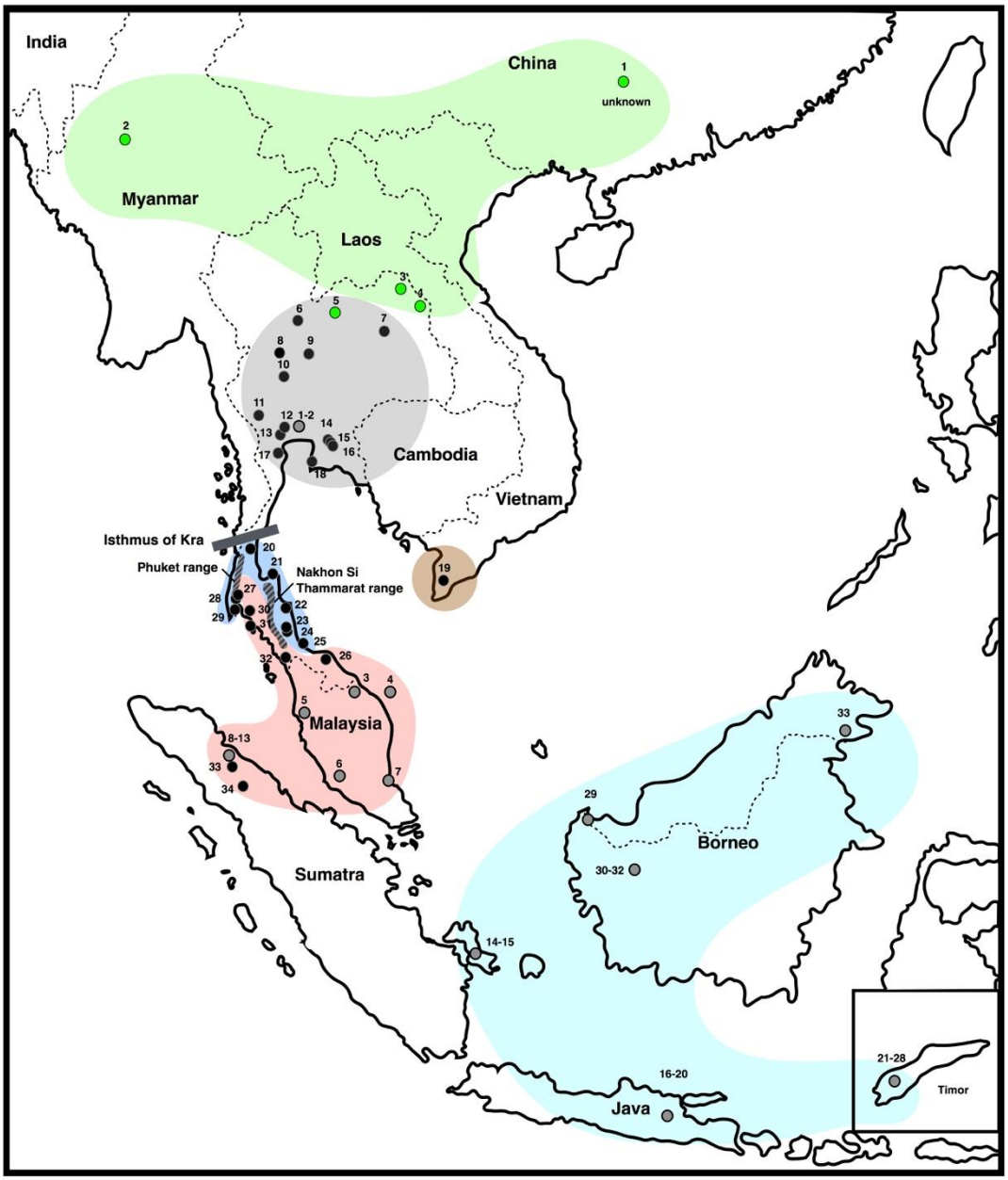

Figure 3.3 Sampling sites of *M. mulatta* (*Mm*) and *M. f. fascicularis* (*Mff*) for the mtDNA analysis. Green, black and grey dots indicate the collected specimens of *M. mulatta*, *M. f. fascicularis* and downloaded sequences, respectively. Shaded areas in green, grey, red, brown, blue, and light blue are for mtDNA subclades of *Mm*, Indochinese *Mff*, Sundaic Andaman Sea Coast *Mff*, Vietnamese *Mff*, Sundaic Thai Gulf *Mff*, and Insular Indonesia *Mff*, respectively.

Figure 3.4 Bayesian phylogenetic tree of mtDNA. Bold letters indicate populations that were also analyzed for *SRY-TSPY* phylogeny (see Figure 3.7-3.8). The green dots after the letters (Mm_WTPMHxxx) indicate that those monkeys were morphologically identified as *Mm*. The value on each branch refers to the posterior probability.

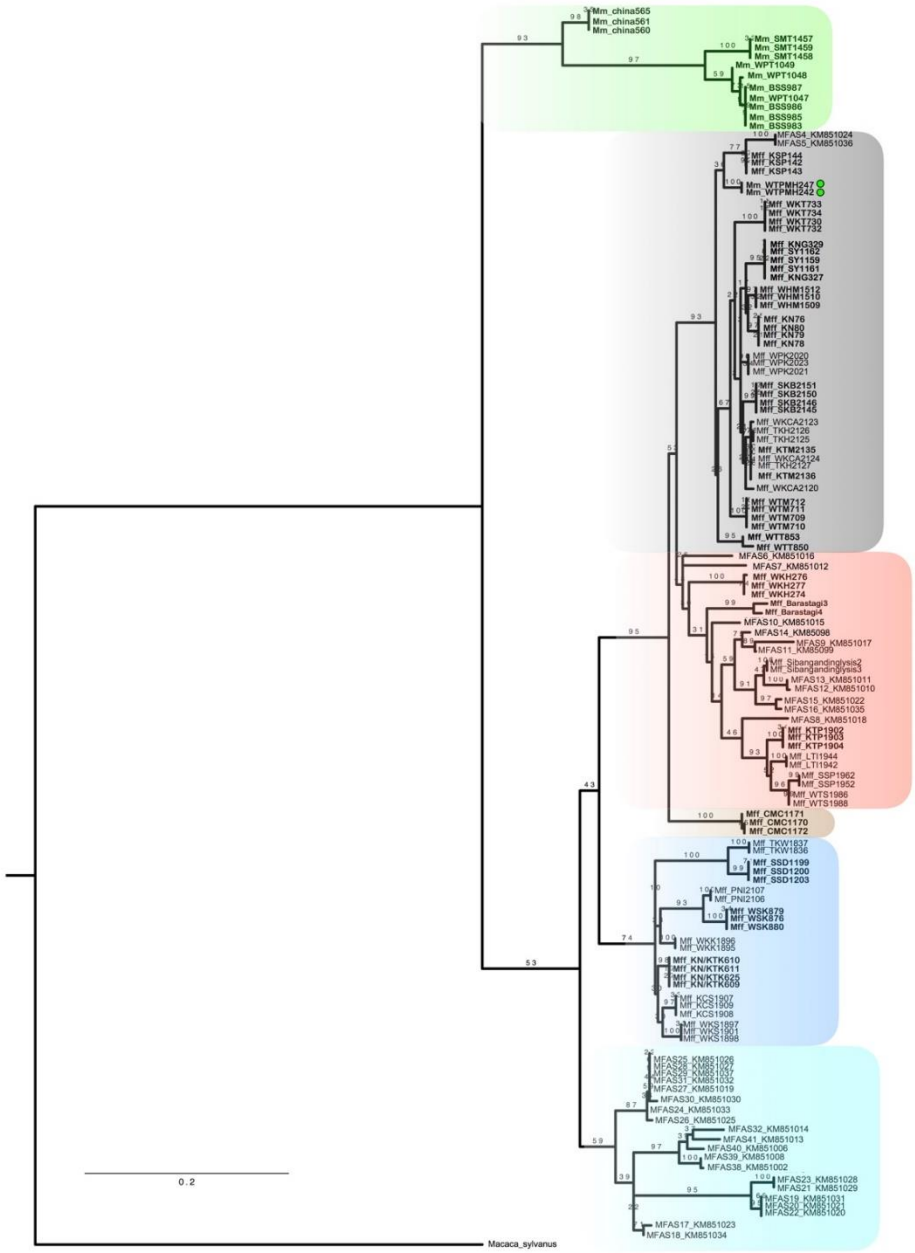

Figure 3. 5 Maximum likelihood phylogenetic tree of mtDNA. Bold letters indicate populations that were also analyzed for *SRY-TSPY* phylogeny (see Figure 3.7-3.8). The green dots after the letters (Mm_WTPMHxxx) indicate that those monkeys were morphologically identified as *Mm*. The value on each branch refers to the bootstrap values.

Divergence time between mtDNA regional subclades

Divergence time was successfully estimated for the relationships among six groups of species/regional subclades (Table 3.5, Figure 3.6); one group of *M. mulatta* and five groups of *M. f. fascicularis* (Insular Indonesia, Sundaic Thai Gulf, Vietnam, Sundaic Andaman Sea Coast and Indochina). First, *M. mulatta* and *M. f. fascicularis* diverged at 1.90 MYA (0.86-3.01 MYA: 95%CI). Within the *M. f. fascicularis* clade, the Insular Indonesian monkeys diverged first from others around 1.19 MYA (0.55-1.92 MYA: 95%CI), followed by the divergence of the Sundaic Thai Gulf subclade at 1.07 MYA (0.50-1.76 MYA: 95%CI). Since no constrain for the tree topology was set, three *M. f. fascicularis* regional subclades (Vietnam, Sundaic Andaman Sea Coast and Indochina) showed different divergence order from the phylogenetic tree inferred above, which reflected rapid divergence among the three subclades. The Sundaic Andaman Sea Coast subclade diverged from the Indochina and Vietnam subclades at 0.73 MYA (0.32-1.18 MYA: 95%CI), and at the end, the Indochinese and Vietnam subclades diverged each other 0.62 MYA (0.28-1.00 MYA: 95%CI).

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Table 3.5 Divergence time between species/regional subclades (in million years ago).

Divergence	Mean	95% Credibility
Msyl / *Mm* + *Mff* *	5.40	4.39-6.40
Mm / *Mff*	1.90	0.86-3.01
Insular *Mff* / other *Mff*	1.19	0.55-1.92
Thai Gulf *Mff* / Andaman + Indochina + Vietnam	1.07	0.50-1.76
Andaman *Mff* / Indochina + Vietnam *Mff*	0.73	0.32-1.18
Indochina *Mff* / Vietnam *Mff*	0.62	0.28-1.00

Msyl = *Macaca sylvanus*; *Mm* = *M. mulatta*; *Mff* = *M. f. fascicularis*;
* = calibration point.

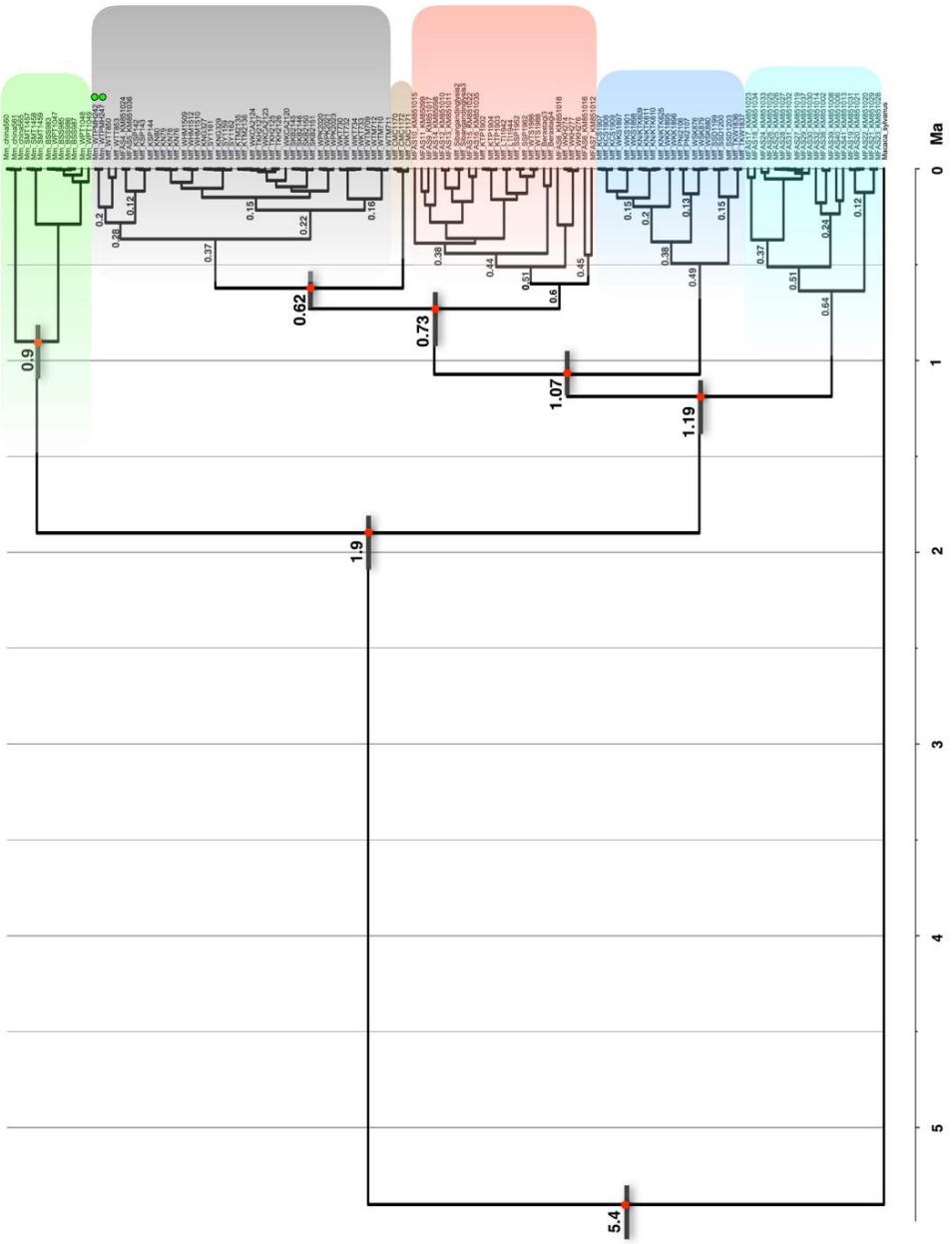

Figure 3. **6** Bayesian inference shows the divergence time estimated based on mtDNA data with 95% confidence interval. The red dot indicates the divergence time among each main clade. Shaded areas in green, grey, brown, red, blue, and light blue are for mtDNA subclades of *Mm*, Indochinese *Mff*, Vietnamese *Mff*, Sundaic Andaman Sea Coast *Mff*, Sundaic Thai Gulf *Mff*, and Insular Indonesia *Mff*, respectively. The green dots after the letters (Mm_WTPMHxxx) indicate that those monkeys were morphologically identified as *Mm*. *Mm* and *Mff* stand for *M. mulatta* and *M. f. fascicularis*, respectively.

Phylogenetic inference based on the *SRY-TSPY* (Y-chromosome) data

Phylogenetic trees of the *SRY-TSPY* sequences from 65 samples of *M. f. fascicularis* and *M. mulatta* constructed by ML and Bayesian analysis showed a same topology, and so only the Bayesian tree is shown in Figure 3.8. All macaques were divided into two main clades. One clade was the *M. mulatta*/northern *M. f. fascicularis* clade which contained all the *M. mulatta* samples (China, SMT, BSS, WPT and WTPMH) grouped with all the Indochinese *M. f. fascicularis* samples (WTT, KSP, KTM, SKB, WTM, WKT, WHM, KN, SY, KNG, CMC, Cambodia, Vietnam and Tum chompol from Thailand), and one Thai gulf population (SSD, no. 20). Their ranges were from 21° 58´ N (SMT) to 9° 56´ N (SSD) latitude (Figure 3.8). The other clade was comprised of only the *M. f. fascicularis* populations (BRT, KTP, KNKTK, WSK, WKH, west Malaysia, east Malaysia, Java, Borneo and south Sumatra) that ranged from 8° 25´ N (WSK) to 3° 12´ N (BRT) latitude (Figure 3.8). Note that the latitudes for the downloaded sequences were not available and so were not counted in this study.

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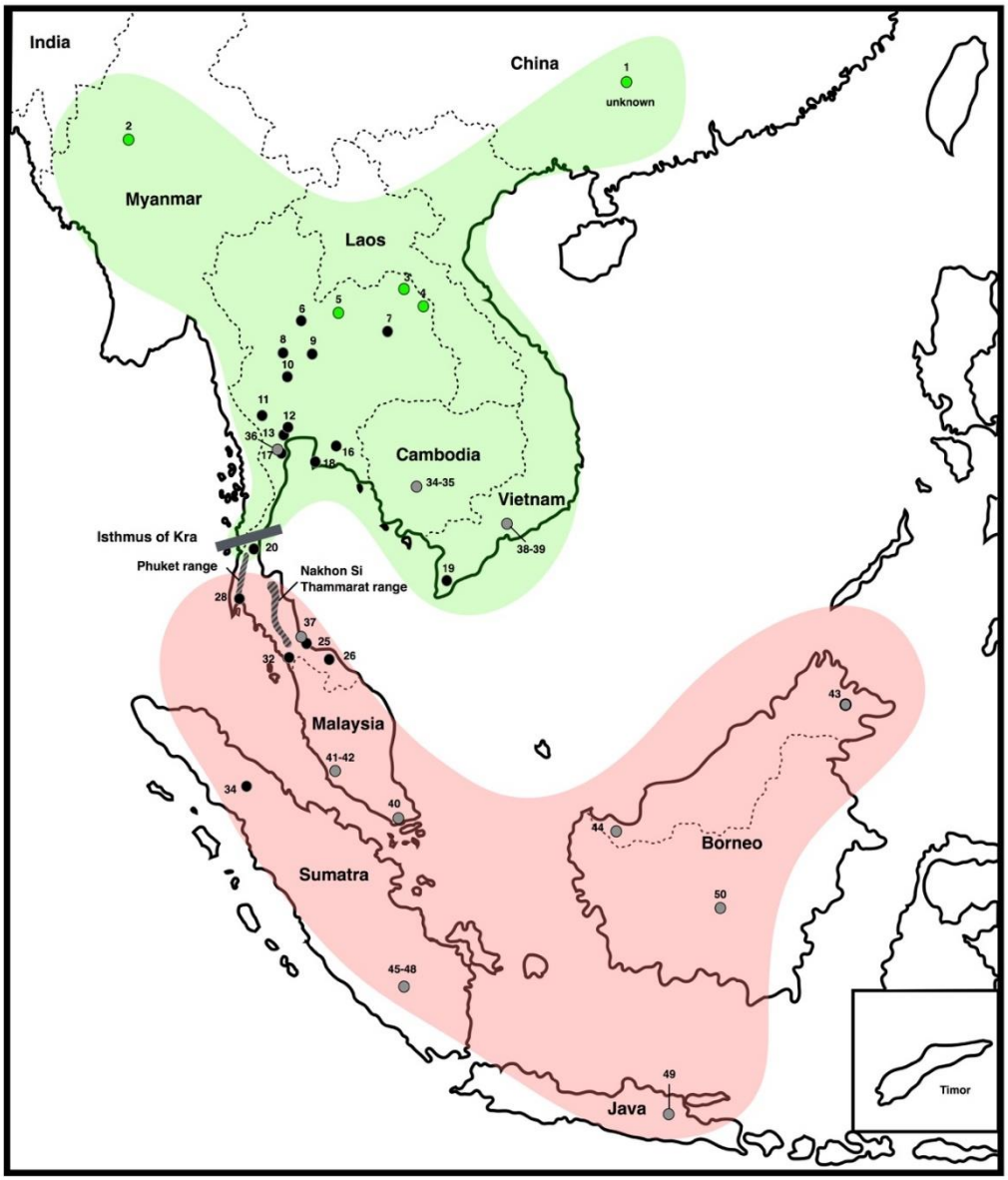

Figure 3. 7 Distribution ranges of *M. mulatta* and *M. f. fascicularis* where green, black and grey dots indicating the collected specimens of *M. mulatta*, *M. f. fascicularis* and downloaded sequences for the *SRY-TSPY* analysis, respectively. Shaded areas in green and red are for *Mm*-like and *Mff*-like clade, respectively. The location numbers in this figure correspond to that in the Table 3.1 and Table 3.3

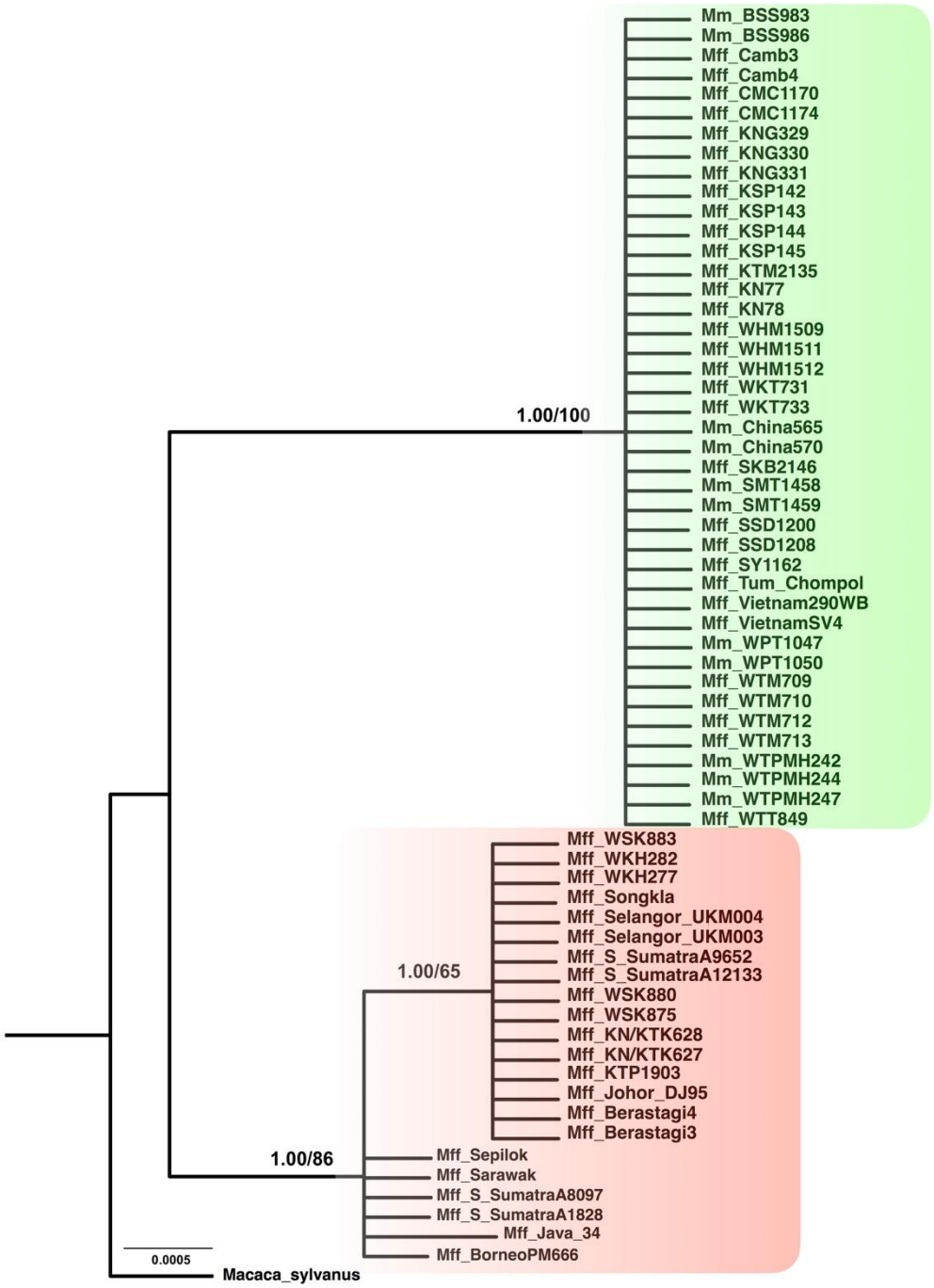

Figure 3.8 Bayesian phylogenetic tree of the *SRY-TSPY* concatenated data. *Mm* and *Mff* stand for *M. mulatta* and *M. f. fascicularis*, respectively. The value on each branch refers to posterior probability/bootstraps value.

Discussion

Although many genetic studies of *M. f. fascicularis* have been performed in the last two decades (Blancher et al., 2008; Kawamoto et al., 2008; Shiina et al., 2010; Kanthaswamy et al., 2013; Abdul-latiff et al., 2014a; Liedigk et al., 2015), only two populations from Thailand located at the center of their distribution range have been analyzed (Tosi et al., 2002, 2003). Thus, the evolutionary history of this subspecies remains obscure. More thorough sampling of *M. f. fascicularis* populations throughout Thailand in this study has started to fill the gap and has shed some light on the hidden evolutionary history of the subspecies.

Genetic differences among the regional subclades of *M. f. fascicularis*

The phylogenetic analysis of mtDNA supports the presence of two major clades among *M. f. fascicularis*, in accord with that reported previously (Harihara et al., 1988; Liedigk et al., 2015). One clade comprised macaques from Indochina, Malay Peninsula and northern Sumatra (continental clade), while the other is comprised of those from other parts of insular Southeast Asia (insular clade). Furthermore, my analysis detected four clearly diverged but previously unknown regional subclades within the continental clade (Sundaic Thai Gulf, Vietnam, Sundaic Andaman Sea Coast and Indochina). The phylogenetic relationships and distribution pattern of these four subclades showed new insights into the evolutionary history of the continental *M. f. fascicularis*.

Although the Sundaic Andaman Sea Coast is located close to the Sundaic Thai Gulf, with both of these regions being located in Sundaic Thailand, the phylogenetic tree showed a closer relationship between the Sundaic Andaman Sea Coast and Indochina/Vietnam subclades than between the Sundaic Andaman Sea Coast and Sundaic Thai Gulf subclades. From this phylogenetic relationship and the estimated divergence time, the following scenario about the evolutionary history of the four subclades can be made. First, after *M. f. fascicularis* expanded throughout Southeast Asia, around 1.19 MYA (0.55-1.92 MYA: 95%CI), gene flow between the Insular and

other regions was restricted. This might be due to the sea level rise in the interglacial period and caused Insular populations to become isolated from those of other regions. Then, the Sundaic and Indochinese monkeys diverged from each other, which may have been induced by the Isthmus of Kra, the largest biogeographical barrier in the region that has influenced many animal and plant species distributions in the area (Denduangboripant & Cronk, 2000; Inger & Voris, 2001; Hughes et al., 2003, 2011). The *M. f. fascicularis* populations which were isolated in the northern side of the Isthmus of Kra later became the founders of the Indochinese, Vietnam and Sundaic Andaman Sea Coast populations, whereas those on the southern side founded the Sundaic Thai Gulf populations. Among the three subclades of the northern side, divergence has occurred rapidly around 0.62-0.73 MYA (0.28-1.18 MYA: 95% CI). This time point corresponds to the earlier interglacials period when the sea level had started to increase at ca. 630,000 years ago (Stirling et al., 2001). Indeed, the close genetic relationship among these three sub regions might occur during the glacial period when the sea level was started to be low (Lambeck, Esat, & Potter, 2002; Stirling et al., 2001; woodruff, 2010) since the early Pleistocene epoch. However, the land had started to be connected (or land bridge) between Indochina and Sunda regions when the sea level was lower than at the present-day up to 60 meters (Sathiamurthy & Voris, 2006) about 1 MYA (Woodruff, 2010). Due to the shallow-water level between southern Vietnam/Cambodia and southernmost Thailand/northern peninsular Malaysia, these areas were connected earlier than the other regions (Sathiamurthy & Voris, 2006 and also Figure 2.6-2.9 in Chapter II). This proposed scenario corresponds with the mtDNA's result of which the WKH, MFAS6 (Kelantan) and MFAS7 (Pulau Redang) *M. f. fascicularis* populations were first diverged from the other Sundaic Andaman Sea Coast *M. f. fascicularis*. Thus, during the glacial period, some *M. f. fascicularis* from southern Indochina (around eastern Thailand, southern Cambodia and Vietnam at present) were favored expansion and emigration by the huge continental shelf of Sundaland. They may have then migrated towards the south-west across the land bridge and colonized the southernmost

part of Thailand (WKH) and northern part of Malay peninsula (MFAS6, MFAS7). After that, some of them migrated southwards and northwards and colonized the northern Sumatra and Thai Andaman Sea Coast, respectively. In the meantime, macaques that lived in the southern side of the Isthmus of Kra (Sundaic Thai Gulf clade) might have been restricted in their distribution range by the Nakhon Si Thammarat mountain range. Therefore, the migration of Andaman Sea Coast subclade to this region (eastern side of Thai peninsula; Thai Gulf side) was hampered.

Female philopatry together with the behavioral characteristics of *M. f. fascicularis* of commonly being found along the forest edge, swamps, coastal, riverbanks and mangrove forests (Fooden, 2006; Gumert, 2011), might account for the separation between the Thai Gulf and Andaman Sea Coast subclades, especially in the mtDNA tree. This scenario could explain the close relationship between the Andaman Sea Coast and Indochina populations yet the distant relationship between the Andaman Sea Coast and Thai Gulf populations (Figure 3.4-3.5).

The scenario of *M. f. fascicularis* migration across the land bridge of Sundaland in the Pleistocene epoch is also supported by previous studies that found a relationship between the Indochina and peninsular Malaysian populations (Abdul-Latiff et al., 2014a) and the Indochina and Indonesian populations (Blancher et al., 2008). Together with the fact that in the glacial period the northern latitude was cooler and drier compared with the equatorial zone, the forest area mainly remained on the Sundaic region, whilst the Indochinese region was largely covered by savanna (Heaney, 1991; Meijaard, 2003). These factors could induce the migration of *M. f. fascicularis* in Indochina towards the south, as proposed above, forming the close relationship between the Indochinese and Andaman Sea Coast *M. f. fascicularis* populations. It might also be possible that the close relationship between these two clades is caused by the incomplete lineage sorting which may occur during the evolution process of the mtDNA.

Based on my phylogenetic tree, WSK and PNI (no. 28 and 29, respectively, in Figure 3.4-3.5 and Table 3.1) are the only two populations in the Andaman Sea Coast

that clustered inconsistently with their origins, because they were grouped within the Thai gulf subclade. Two possible explanations are proposed. Considering their distribution, it is possible that these two populations migrated across Sundaic Thailand (from the Thai Gulf to the Andaman Sea Coast side) because the WSK and PNI populations occupy the regions between the two marginal Phuket and Nakhon Si Thammarat mountain ranges, which were considered as a part of Tanintharyi mountain hills in the classical reference (Gupta A, 2005), without any zoogeographical barrier between these areas. Alternatively, they were subjected to human translocation. Although this has not been reported in many cases, it has been reported that some monkeys were caught and then released into another troop, both in terms of conspecific and heterospecific taxa (Malaivijitnond et al., 2007; Malaivjitnond & Hamada, 2008).

Male-mediated gene flow from *M. mulatta* into *M. f. fascicularis* populations

The *SRY-TSPY* based phylogenetic tree suggests male-mediated gene flow. Within the *SRY-TSPY* phylogeny, all of the sequences were divided into two clades, (i) *M. mulatta* together with the Indochinese *M. f. fascicularis* and (ii) Sundaic *M. f. fascicularis*, whereas in the mtDNA phylogenetic tree *M. mulatta* and *M. f. fascicularis* showed a reciprocally monophyletic relationship with respect to each other. These results agree with previous reports about hybridization between these two species (Tosi et al., 2002; Hamada et al., 2006; Malaivijitnond et al., 2008; Kanthaswamy et al., 2008; Barr et al., 2011) being driven by male *M. mulatta* invading into *M. fascicularis* populations (Bonhomme et al., 2009; Stevison & Kohn, 2009). Among my sampled populations in the *SRY-TSPY* analysis, SSD (no. 20 in Table 3.1 and Figure 3.7) was the southernmost population (9° 56´ N, 99° 02´ E) that was grouped in the *M. mulatta*/Indochinese *M. f. fascicularis* clade, whereas WSK (no. 28 in Table 3.1 and Figure 3.7) was the northernmost population (8° 25´ N, 98° 28´ E) that was grouped within the Sundaic *M. f. fascicularis SRY-TSPY* clade. This suggests that gene flow from *M. mulatta* southwards into *M. f. fascicularis* populations was limited somewhere between

the SSD and WSK populations. If so, this partially confirms the hypothesis that the Klong Marui fault/Isthmus of Kra limits the gene flow from *M. mulatta* (Tosi et al., 2002). However, the SSD population is located on the southern side of the Isthmus of Kra, and so male-mediated gene flow from *M. mulatta* would have had to pass through some part of the isthmus. Since the Isthmus of Kra is not a sharp restrictive barrier, but rather causes gradual changes in species distribution in many taxa (Hughes et al., 2011), it is not surprising that some male monkeys who carried genetic material of *M. mulatta* passed southwards and reached the SSD population. However, these monkeys may have not been able to migrate further because of the habitat limitation of the forest type. Interestingly, the SSD population was grouped with the Sundaic Thai Gulf subclade in the mtDNA phylogeny, which suggests that the biogeographic barrier has worked differently between sexes, presumably due to the sex-dependent differences in their migration patterns (male dispersal and female philopatry). Nevertheless, Y-chromosome (*SRY* and *TSPY*) markers in this study are functional genes and showed low genetic diversity. Therefore, only two main clusters (*M. mulatta* together with the Indochinese *M. f. fascicularis* and Sundaic *M. f. fascicularis*) were shown here. Increasing the number of genetic markers with higher polymorphism could magnify the hybridization scenario between *M. mulatta* and *M. f. fascicularis*.

No population that possessed the mtDNA of the Sundaic Andaman Sea Coast subclade was found to possess the Y-chromosome of the *M. mulatta*/Indochinese *M. f. fascicularis* clade. This suggests that the introgression from *M. mulatta* into *M. f. fascicularis* started after the divergence of the Indochinese/Vietnam and Sundaic Andaman Sea Coast subclades, a divergence time that has been estimated at around 0.73 MYA (0.32-1.18 MYA: 95%CI). However, the gene introgression/migration time estimates vary and range from over 1 MYA by autosomal single nucleotide polymorphism (SNP) analysis (Osada et al., 2010) to 3,400 years by nuclear microsatellite analysis (Bonhomme et al., 2009). In fact, the true time frame of hybridization or even divergence time between these two species may be difficult to

estimate due to their complicated evolutionary history, especially between the *M. f. fascicularis* and *M. mulatta* distributed in the Indochina region. They share many polymorphic alleles, but it is difficult to distinguish whether they are derived from recent common ancestry or hybridization (Stevison & Kohn 2009; Osada et al., 2010). Therefore, further genomic scale studies focusing on monkeys from wide origins are required to uncover the details of the hybridization.

Morphological characteristics of the WTPMH population

This study determined the genetic characteristics of many populations of *M. mulatta* and *M. f. fascicularis* living in Thailand and nearby countries, including those in the hybrid zone. All macaque populations were found to possess mtDNA related to their species/subspecies identification using the morphological/distribution criteria except for one *M. mulatta* population (WTPMH). Even though the macaques in the WTPMH population showed *M. mulatta*-like morphological characters (bipartite pelage color and a $57.0 \pm 4.2\%$ RTL (Malaivijitnond et al., 2007)), they were grouped with *M. f. fascicularis* in the mtDNA phylogeny. This suggests that WTPMH monkeys possess mixed genetic characteristics between *M. mulatta* and *M. f. fascicularis*, and probably their genetic composition mainly reflects past genetic influx of *M. mulatta*. According to this result, the WTPMH population is an attractive model to study the historical population dynamics and gene flow between these two taxa.

Phylogeography of *M. f. fascicularis* populations throughout Thailand and the vicinity

By investigating a large number of *M. f. fascicularis* populations from the center of their distribution range (Thailand), a more complete phylogeography of the subspecies was revealed and was much more complicated than previously reported. The presence of four mtDNA subclades within the continental clade suggests that several

zoogeographic factors, such as the Sundaland land bridge, the Isthmus of Kra, and the Nakhon Si Thammarat and Phuket mountain ranges, have affected the population history of the subspecies. In addition, sequence analysis of the *SRY* and *TSPY* genes on the Y-chromosome compared to the mitochondrial sequences revealed that male-mediated gene flow from *M. mulatta* to *M. f. fascicularis* was limited between the SSD (9° 56′ N) and WSK (8° 25′ N) populations, which are located south of the well-known Isthmus of Kra (approximately 10° 30′ N). This study highlighted the importance of investigating *M. f. fascicularis* within Thailand and the vicinity to elucidate the evolutionary history of this subspecies. Further studies that include more DNA samples, especially from Thailand, together with the use of more powerful markers, such as SNPs, would help us to gain new insights into the evolutionary history of *M. f. fascicularis*.

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CHAPTER IV
A NEW PERSPECTIVE OF HYBRIDIZATION BETWEEN *Macaca fascicularis fascicularis* AND *M. mulatta* IN THEIR NATURAL HABITATS: ISTHMUS OF KRA IS NOT A TRUE ZOOGEOGRAPHICAL BARRIER

Introduction

M. mulatta and *M. fascicularis fascicularis* occupy wide geographic ranges and are well-adapted to many habitat types. *M. mulatta* inhabit ranges between *ca.* 15 to 36° N in South and Southeast Asia encompassing the countries of Afghanistan, Pakistan, Bangladesh, Bhutan, India, Nepal, China, Myanmar, Thailand, Laos and Vietnam (Fooden, 2000). Their northern range is defined by the Great Indian desert, the Tibetan Plateau and the transition between mesothermal and microthermal climates (Fooden, 2000), and their southern limit is restricted by interspecific competition with *M. f. fascicularis*. *M. f. fascicularis* are distributed throughout the mainland and insular regions of Southeast Asia between *ca.* 20° N and 10° S (Fooden, 1997) and includes the Philippines, Indonesia, Malaysia, Myanmar, Thailand, Laos, Cambodia, Vietnam and the lesser Sunda island as far as east Timor and Nicobar island (India) (Fooden, 2000). Compared with other animal models such as rodents, with which humans share a common ancestor that lived approximately 60 MYA (Benton & Donoghue, 2007), macaques share common ancestry with humans dating to approximately 25 MYA (Glazko & Nei, 2003). As such, humans and macaques share many physiological, anatomical and immunological traits with each other (Bontrop, 2001; Jerome & Peterson, 2001; Kennedy, Shearer, & Hildebrand, 1997).

Due to the wide geographic ranges of *M. mulatta* and *M. f. fascicularis* and their sharing of many traits with humans, these two species are the most commonly encountered macaque species. Consequently, both species have been used as a primate

model in biomedical research, especially after the *M. mulatta* and *M. fascicularis* draft genome sequences were published (Gibbs et al., 2007; Higashino et al., 2012). However, one important issue of concern before selecting any animal as a translational model of human disease is the genetic characteristics of the model species that can influence experimental results. For example, *Plasmodium knowlesi*, a causative agent for human malaria, produces a virulent infection in *M. mulatta*. However, *M. fascicularis*, the natural host of this parasite, exhibits no severe manifestation of disease after being infected (White, 2008). Therefore, *M. mulatta* are more suitable subjects for the study of malaria in humans, because they share more post-infection symptoms with humans than do *M. fascicularis*. On the other hand, *M. fascicularis* are more commonly used as an animal model for drug toxicity (Iwasaki & Uno, 2009). In addition to species-specific differences, subspecies or regional differences can also influence the suitability of animal models for the study of any particular disease. For example, Indian *M. mulatta* are a more suitable translational model for the study of HIV/AIDS in humans than Chinese *M. mulatta*, because they are more susceptible to SIV than their conspecific from China (Joag et al., 1994).

M. mulatta and *M. f. fascicularis* live closely in the parapatric area of their natural habitats and have become hybridized. Their hybrid zone was proposed to lie between 15 and 20° N, covering four of the countries that comprise Indochina: Thailand, Myanmar, Laos and Vietnam (Fooden, 1995; Fooden, 2000; Hamada et al., 2006). Because hybrid offspring are fertile, subsequent mating of these hybrids with full-blood *M. mulatta* or *M. f. fascicularis*, or their hybrids, have led to a broad range of admixture proportions among macaques in Indochina. In addition to its influence on the suitability of hybridized animals as research subjects, hybridization is an evolutionary mechanism that has been widely studied based on morphological, physiological and genetic analysis (Tosi et al., 2002; Hamada et al., 2008; Malaivijitnond et al., 2008). Morphological characteristics of *M. mulatta*/*M. f. fascicularis* hybrids have been examined both in wild and captive troops (Hamada et al., 2008; Hamada et al., 2006;

Jadejaroen et al., 2015). Their genetic composition and levels of admixture have been determined using mtDNA, Y-chromosome, STRs and SNPs markers (Kanthaswamy et al., 2008; Osada et al., 2010; Satkoski et al., 2013; Tosi et al., 2002). Although many studies have identified hybridization between these two species for more than a decade, some issues remain unresolved. For instance, Tosi et al. (2002) proposed the unidirectional hybridization model of *M. mulatta* male introgressing to *M. f. fascicularis* groups. Bonhomme et al. (2009) analyzed Indochinese *M. f. fascicularis* along with Chinese *M. mulatta* and agree that only unidirectional gene flow from *M. mulatta* to *M. f. fascicularis* populations occurred. These results conformed with Stevison & Kohn (2009) who examined captive *M. f. fascicularis* and *M. mulatta* samples from various regions and suggested that genetic introgression from *M. mulatta* to *M. f. fascicularis* population was restricted to mainland Indochina. Other research suggests that hybridization should occur bi-directionally, both from *M. mulatta* to *M. f. fascicularis* and from *M. f. fascicularis* to *M. mulatta* (Hamada et al., 2016; Kanthaswamy et al., 2010; Osada et al., 2010). *M. mulatta* gene flow to *M. f. fascicularis* that extends far beyond Indochina and southward to Sundaland and *M. f. fascicularis* to *M. mulatta* gene flow occurring eastward to eastern China and India has been proposed (Kanthaswamy et al., 2010; Osada et al., 2010).

Considering the hybrid ranges, Thailand, Myanmar, Laos and Vietnam are likely key for the investigation due to their location at the center of the hybrid zone. Thailand connects the distribution ranges of *M. mulatta* (to the north) and *M. f. fascicularis* (to the south) and encompasses the geographical regions of both Indochina and Sunda. In this study, macaque populations from mainland Southeast Asia, including Myanmar, Vietnam, Laos, Cambodia, and Thailand except for its southern peninsular part, are referred to as Indochinese, while the southern population from peninsular Thailand, Malaysia and Sunda (Sumatra, Java, Borneo, Bali, and Lesser Sunda Islands until Timor Island) are referred to as Sundaic. Heretofore, most previous

studies have been performed on *M. f. fascicularis* of either unknown origin or those known to have originated in a particular country [e.g., Malaysia, Indonesia, the Philippines and Indochina (combined between Vietnam and Cambodia)] and *M. mulatta* known only to have originated in some unknown site in China or India, limiting an explanation of the complete hybridization scenario. Only recently, in Chapter III has studies of genetic characteristics of *M. mulatta* and *M. f. fascicularis* originating in precisely known locations throughout Thailand and its vicinity identified the strong impact of biogeographical factors, such as the Isthmus of Kra, the Tanintharyi ranges and ancient land bridges, on genetic differences among populations of *M. f. fascicularis* (Bunlungsup et al., 2017). This study revealed a more complicated hybridization scenario than originally thought. However, previous study was based on uni-parental markers that traced either solely maternal (mtDNA) or solely paternal (Y-chromosome) inheritance rather than bi-parental markers which can trace in greater detail both paternal and maternal contributions.

In this study, specimens of *M. f. fascicularis* and *M. mulatta* covering the suspected areas of the hybridization were analyzed. Samples of precisely known origins in Thailand, Myanmar and Laos, along with reference populations of *M. mulatta* from China and *M. fascicularis* populations from Indochina (Vietnam/ Cambodia), Singapore/ Sarawak, Sumatra/ Indonesia Luzon/ Zamboanga and Mauritius were investigated. The aim of this study is to examine how hybridization has altered the genetic composition of *M. mulatta* and *M. f. fascicularis* populations and how their genetic admixture proceeds from the hybrid zone outward using SNPs. This objective is to shed additional light on the evolutionary process and implications of hybridization and its consequences for biomedical research.

Methods

Sample collection and species identification

Blood samples were derived from DNA Bank of the Primate Research Unit, Chulalongkorn University. Each of four populations of *M. mulatta* and *M. f. fascicularis* occupying a natural habitat in Thailand, Myanmar and Laos that includes their proposed hybrid zone (Fooden, 1964; Hamada et al., 2016) (Table 4.1) were temporarily trapped and sampled as previously described (Malaivijitnond & Hamada, 2008; Malaivijitnond et al., 2008). The species status of the monkeys in each population was identified based on two criteria (known distributional ranges and morphological characteristics) as mention earlier in Chapter III. According to these characteristics, monkey populations in the proposed hybrid zone (15-20° N) exhibiting mixed morphological characteristics were identified as predominantly either *M. mulatta* or *M. f. fascicularis*.

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Faculty of Science in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University, Protocol Review No. 1423010.

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Table 4. 1 Region, locality and geographical coordinate (GPS) of the sampling sites of wild caught *M. mulatta* and *M. fascicularis*. Dash line separates *M. mulatta* (upper) and *M. fascicularis* populations (lower). IOK = Isthmus of Kra.

Taxon	Region	Name of location	GPS
M. mulatta	China	1. Yunnan/Myanmar border	–
		2. Wuhan	–
		3. Suzhou/Kunming*	–
	Myanmar	4. Shin Ma Taung (SMT)	21° 58´ N, 95° 10´ E
	Thailand	5. Bann Sang School (BSS)	17° 51´ N, 103° 57´ E
		6. Wat Tham Pa Mak Ho (WTPMH)	17° 14´ N, 101° 46´ E
	Loas	7. Ban Dong Muang (BDM)	16° 54´ N, 105° 25´ E
M. fascicularis	Thailand	8. Wat Haad Moon (WHM)	16° 51´N, 100° 28´ E
	(North of IOK)	9. Wat Khao Thamon (WKT)	13° 02´ N, 99° 57´ E
	Thailand	10. Suan Somdet Prasrinakharin	9° 56´ N, 99° 02´ E
	(South of IOK)	Chumphon (SSD)	
		11. Khao Noi/Khao Tang Kuan (KN/KTK)	7° 12´ N, 100° 35´ E
	Indochina	12. Vietnam/Cambodia	–
	Sunda	13. Singapore/Sarawak	–
		14. Sumatra/Indonesia	–
		15. Luzon/Zamboanga	–
		16. Mauritius	–

*The Suzhou/Kunming samples were obtained from the California National Primate Research Center (CNPRC). These animals are descendants of animal imported from China.

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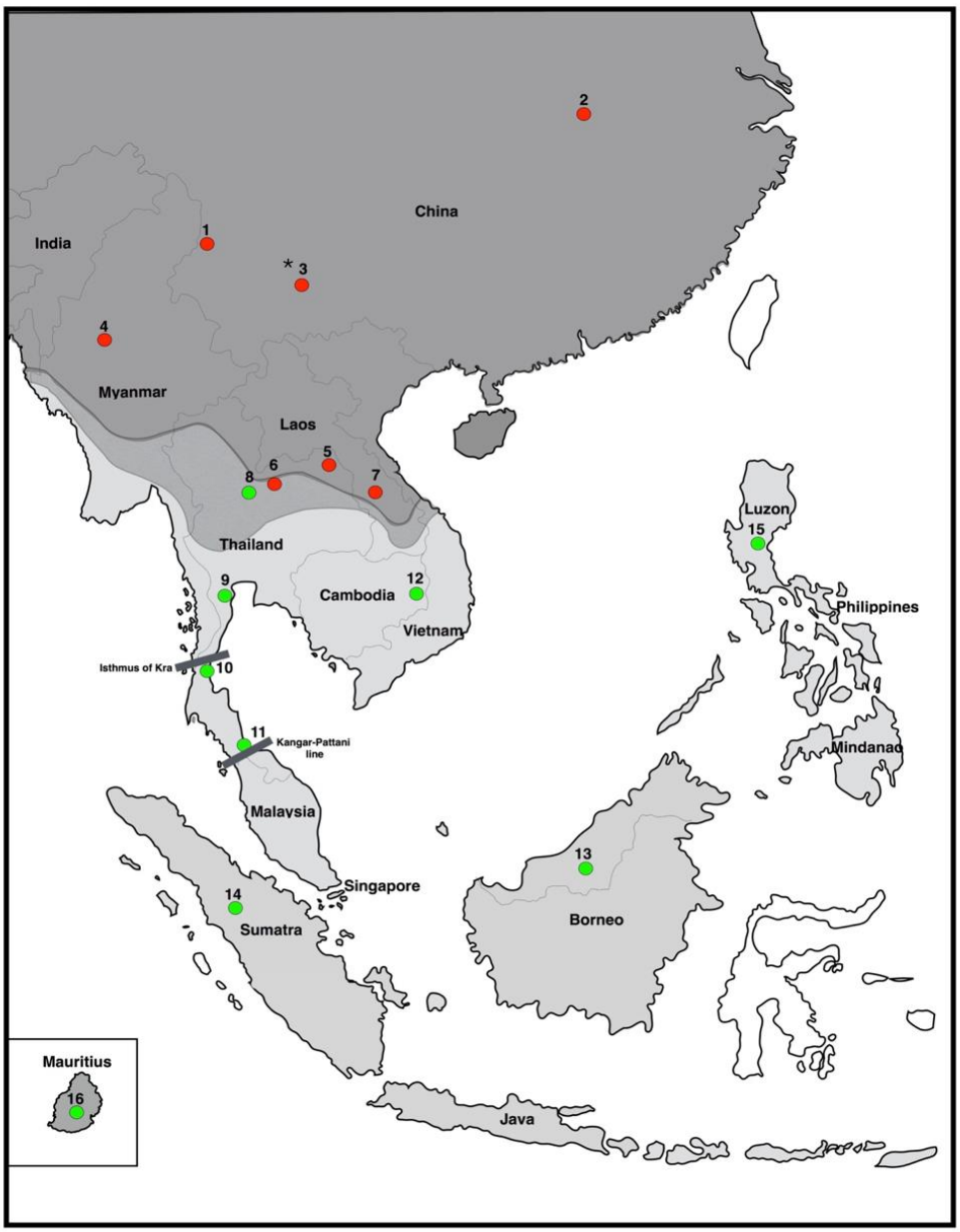

Figure 4.1 Distribution ranges of *M. mulatta* (dark gray), *M. fascicularis* (light gray), and their hybrid zone (gray). Red and green dots indicate the collected specimen of *M. mulatta* and *M. fascicularis*, respectively. The location numbers in Figure 1 correspond to those in the Table 1. "*" references the Suzhou/Kunming samples, cited at Kunming on the map, which were obtained from the California National Primate Research Center (CNPRC).

SNP genotyping

DNA samples of wild *M. mulatta* and *M. f. fascicularis* were collected from populations in the following eight locations: Shin Ma Taung (SMT; n = 26), Bann Sang School (BSS; n = 30), Wat Tham Pa Mak Ho (WTPMH; n = 19), Ban Dong Muang (BDM; n = 17), Wat Haad Moon (WHM; n = 30), Wat Khao Thamon (WKT; n = 26), Suan Somdet Prasrinakharin Chumphon (SSD; n = 11) and Khao Noi/Khao Tang Kuan (KN/KTK; n = 29) (Table 4.1). These DNA samples were adjusted to a concentration of 60-100 ng/µl and genotyped for 96 SNPs (Table 4.2) comprising the Admixture and Ancestry panels described in Zhang et al. (2017). Ten µl of each sample were loaded onto 96-well plates and submitted to the DNA Technologies Core of the University of California Davis Genome Center for genotyping using the Fluidigm Assay on a Fluidigm EP1 system. Specific target amplification (STA) was applied to reduce nonspecific product.

To identify hybridization, genotyping data of references *M. mulatta* and *M. fascicularis* living outside the hybrid zone were also included in this study. Chinese *M. mulatta* from the Yunnan/Myanmar border (n = 17), Wuhan (n = 28) and Suzhou/Kunming (descendants of animals housed at the California National Primate Research Center, CNPRC; n = 25) were used as a reference for *M. mulatta*, and *M. fascicularis* from Vietnam/Cambodia (n = 23), Singapore/Sarawak (n = 36), Sumatra/Indonesia (n = 38), Luzon/Zamboanga (n = 43) and Mauritius (n = 24) were included as a reference for *M. fascicularis*. Note here that Luzon/Zamboanga populations are *M. f. philippinensis* mixed with *M. f. fascicularis*. Only SNPs providing genotypes for at least 90% of all samples and samples providing genotypes for at least 90% of all SNPs were employed in further analysis.

Population genetic parameters and genetic structure analysis

Genotypes of all population samples were tested for their fit to Hardy-Weinberg Equilibrium (HWE) expectations, and observed heterozygosity (OH), expected heterozygosity (EH), pairwise F_{ST}, the inbreeding coefficient (F_{IS}) and minor allele frequency (MAF) were estimated for each population sample using Arlequin version 3.5.2.2 (Excoffier & Lischer, 2010).

STRUCTURE version 2.3.4 (Pritchard et al., 2000) was used to characterize the admixture between the two species. To estimate the proportion of genetic admixture, all *M. mulatta* and *M. fascicularis* data were analyzed based on the admixture panel of 48 SNPs assuming an admixture model. 500,000 iterations of MCMC were run, and 20% (100,000 iterations) of data were discarded as burn-in. The number of population clusters (K) was set between 2 to 12 and run for 5 iterations for each value of K. To calculate the highest probability of the true number of clusters, a deltaK analysis based on the second order derivation of the change in variance of the log probability between successive K values (Evanno et al., 2005) was performed over 5 iterations. Genetic differentiation among *M. fascicularis* populations was assessed using only the *M. fascicularis* genotypes based on the 48 SNP ancestry panel. All parameters were set as for the analysis described above using the admixture panel, and deltaK was calculated to find the value of K with the highest probability.

Results

SNP genotyping data

All 96 SNPs together with estimates of their genetic diversity are shown in Table 4.2. Eighty of the 96 SNPs provided 90% or greater complete genotype profiles for the 422 samples, all but seven of which yielded genotypes for at least 90% (i.e., 86) of the 96 SNPs. The 16 SNPs and 7 samples providing fewer than 90% complete profiles were eliminated from further analysis, providing a final data set of 80 SNPs (41 SNPs

for admixture and 39 SNPs for ancestry) with genotypes assigned to 415 individuals (162 *M. mulatta* and 253 *M. fascicularis*). All 80 loci across 16 populations featured only one (monomorphic) or two alternate alleles (dimorphic) with a mean of 1.542.

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Table 4. 2 All 96 loci name and their estimated genetic diversity (expected heterozygosity).

Loci name	Population name																Mean
	Yunn/ Myan	Wuhan	Suzh/ Kunm	SMT	BSS	WTP MH	BDM	WHM	WKT	SSD	KNK TK	Viet/ Camb	Mala/ Sing	Suma/ Indo	Luzon	Maur	
262799097	0.4706	0.5084	0.5102	-	-	0.5007	0.1141	-	0.1560	0.4790	-	0.3217	-	-	-	-	0.1913
262799857	0.1658	0.4442	0.3265	0.3167	0.4808	0.4225	0.5080	-	0.0417	-	-	0.0435	-	-	-	-	0.1719
262800426	0.3369	0.4156	0.3265	-	-	0.2347	-	-	-	-	-	-	-	-	-	-	0.0821
262801490	0.1141	0.1656	0.1502	0.0754	0.4271	0.3983	0.4706	-	0.2544	-	-	0.3594	-	-	-	0.0417	0.1536
262802570	0.1658	-	0.1837	0.5098	0.5079	0.1494	0.5080	-	0.0417	-	0.4672	0.1981	-	-	-	-	0.1707
262799114	0.2139	0.2987	0.1906	-	-	0.3983	-	-	-	-	-	0.1981	-	-	-	-	0.0812
262799872	0.4706	0.5031	0.5094	-	0.3452	0.3983	0.4991	0.5034	0.0417	-	-	0.0850	-	-	-	-	0.2097
262800683	0.2139	0.3429	0.1151	0.2376	-	0.2731	0.2995	-	0.0417	-	-	0.1623	-	0.0278	-	-	0.1071
262801617	0.0588	0.2013	0.2457	-	-	0.5007	-	-	0.2837	0.3368	-	0.2937	-	-	-	-	0.1201
262802258	0.1141	0.0701	0.1151	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0187
262802748	0.1658	0.1948	0.2743	0.5098	0.5079	0.2731	0.4278	-	-	-	0.5102	0.4957	0.5067	0.5055	0.5048	0.5027	0.3362
262799228	0.4866	0.4156	0.4580	-	0.2350	0.4623	0.5080	0.1831	0.0816	-	-	0.0850	-	-	-	-	0.1822
262799959	0.4510	0.3994	0.4441	0.3401	0.4944	0.5078	0.1658	0.5062	-	-	-	0.2638	-	-	-	-	0.2233
262800830	0.4980	0.4987	0.4965	-	0.4130	0.4225	0.2995	-	0.0417	-	-	0.0850	-	-	-	-	0.1722
262802764	0.2995	0.2227	0.4441	0.4910	0.0655	0.5007	0.5152	0.3452	0.2544	-	-	0.2319	-	-	-	-	0.2106
262800900	0.3708	0.2747	0.4286	-	-	0.2347	-	0.5034	-	-	-	0.3720	-	-	-	-	0.1365
262802358	0.1141	0.1351	0.1837	-	0.3452	0.1494	0.1141	0.5034	0.0417	-	-	0.1623	-	-	-	-	0.1093
262802765	0.3145	0.4857	0.4870	0.4344	0.4987	0.3857	0.5080	0.4944	0.1300	0.1895	0.0400	0.2937	-	-	-	-	0.2664
262799373	0.5152	0.4305	0.5069	0.4012	0.3638	0.5121	0.4278	0.4944	0.2544	0.2684	0.2155	0.4763	0.3001	0.0278	-	-	0.3247
262800189	0.3708	0.2227	0.3502	-	0.5079	0.4225	0.2585	0.4994	0.1560	-	0.5094	0.4329	0.0548	-	-	0.0417	0.2392
262801017	0.5080	0.4156	0.4702	0.2919	0.5079	0.4438	0.4866	0.4944	0.5027	-	0.4220	0.1300	-	-	-	0.1197	0.2995
262801871	0.4706	0.5091	0.5069	0.3620	0.5079	0.4438	0.2139	-	0.5027	0.4421	0.2457	0.4145	-	-	-	-	0.2887
262802390	0.4436	0.4565	0.4971	0.2919	0.2593	0.4908	0.5134	-	-	-	0.1560	0.3478	0.1826	-	-	-	0.2274
262802774	0.0588	-	0.1151	-	0.4944	0.2731	-	0.4627	0.3942	0.4421	0.2155	0.4763	0.0810	0.1549	-	0.0417	0.2006
262799566	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0000
262800202	0.0588	0.1351	0.3012	-	0.5079	0.3414	0.1141	-	-	0.4790	-	-	-	-	-	-	0.1211
262801294	-	0.4442	0.3265	0.2655	0.0655	0.4623	0.2139	0.4401	0.2937	0.1895	0.1151	0.5111	0.1064	-	-	-	0.2146
262801948	0.4011	0.4929	0.4441	-	0.4520	0.0526	0.2995	-	-	0.1000	-	-	0.0278	-	-	-	0.1419
262802803	0.4510	0.4987	0.4965	0.3824	0.4944	0.3983	0.4011	0.5085	-	0.1000	-	0.2738	0.0810	0.0278	-	-	0.2571
262799689	0.4011	0.5033	0.3012	0.3167	0.0966	0.3414	0.3369	-	0.4885	0.1000	0.5069	0.3720	-	-	-	-	0.2353
262800404	0.4866	0.2987	0.3722	0.1772	0.5034	0.5078	0.5080	0.2096	0.4965	-	-	0.3942	-	-	-	-	0.2471

Loci name	Population name																Mean
	Yunn/	Wuhan	Suzh/	SMT	BSS	WTP	BDM	WHM	WKT	SSD	KNK	Viet/	Mala/	Suma/	Luzon	Maur	
262801360	0.2139	0.4565	0.3927	0.3620	0.2096	0.0526	0.1658	-	-	0.2684	-	0.2319	-	-	-	-	0.1471
262801978	-	-	-	-	-	-	-	-	0.2410	0.3368	-	0.3478	-	-	-	-	0.0579
262802486	0.4706	0.4675	0.4808	0.5091	0.0333	0.3087	0.1754	0.3977	0.1981	-	-	0.2937	-	-	-	-	0.2084
262802917	0.3708	0.4305	0.2743	-	0.4881	0.2731	0.5080	-	-	-	-	0.0435	-	-	-	-	0.1493
262799818	0.0588	0.0357	0.2457	-	0.3814	0.0526	0.0588	-	0.4034	-	-	0.4145	0.0278	-	-	-	0.1049
262800420	0.5141	0.4929	0.4965	0.3401	0.4401	0.4623	0.5152	0.3452	0.0417	-	0.2155	0.2937	-	-	-	0.0417	0.2624
262801479	0.5134	0.5033	0.4286	-	0.1831	0.3414	0.5134	0.4994	-	-	0.3927	0.5111	0.5067	0.5068	0.5034	0.5027	0.3691
262802032	0.2995	0.4675	0.5069	0.4344	0.5034	0.4438	0.2585	-	-	-	-	0.1981	-	-	-	0.0417	0.1971
262802569	0.5081	0.5066	0.5027	0.5098	0.5085	0.5135	0.5152	0.5085	0.5106	0.5263	0.4898	0.5073	0.5068	0.5035	0.4949	0.5027	0.5072
262802938	0.2585	0.4565	0.2743	0.3824	0.3977	-	0.4278	0.4627	0.4145	0.5211	0.1560	0.3217	-	-	-	-	0.2546
SNP_at_chr1_16079968	0.4278	0.1656	0.3265	0.4827	0.4723	0.4225	-	0.3452	0.1246	0.1895	0.0784	-	-	0.0810	0.1896	-	0.2066
SNP_at_chr1_49110258	-	-	-	0.4977	0.0655	-	-	-	0.2837	0.2684	0.5027	-	0.0810	0.0278	-	0.0417	0.1105
SNP_at_chr1_66911428	-	-	-	-	-	0.0526	-	-	-	-	0.1502	-	0.1311	0.4304	-	0.1197	0.0553
SNP_at_chr10_5238374	0.5152	0.5091	0.5106	0.5098	0.5085	0.5135	0.5152	0.5085	0.5106	0.5263	0.5102	-	-	0.1064	0.4071	-	0.3844
SNP_at_chr10_5310803	-	-	-	-	-	-	-	-	-	0.4421	0.3265	-	0.1064	0.0810	0.1513	-	0.0692
SNP_at_chr10_5701898	0.5081	0.3994	0.5094	0.4971	0.4483	0.3369	0.4706	0.4483	0.3830	0.1111	0.0417	0.3330	0.2331	0.3068	0.2758	0.4787	0.3613
SNP_at_chr11_4110282	0.5080	0.5091	0.5102	-	-	0.1494	0.1210	0.0333	0.2937	0.1895	0.3942	0.1623	0.5067	0.4973	0.2758	0.4539	0.2878
SNP_at_chr11_4112285	0.5152	0.5102	0.1359	0.5098	0.5085	0.0526	0.4011	-	0.3830	0.3368	0.2457	0.2319	0.1311	-	-	-	0.2476
SNP_at_chr11_5757914	-	-	-	-	-	-	-	-	-	-	-	0.1300	-	-	-	0.5027	0.0395
SNP_at_chr11_7015538	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0548	0.3852	-	0.0275
SNP_at_chr12_5369712	0.5152	0.5091	0.5102	-	-	-	-	-	-	-	-	0.2937	0.4930	0.3940	-	0.1197	0.1772
SNP_at_chr12_7287095	-	0.3913	0.0000	-	-	0.1024	-	0.0966	0.0850	0.4790	0.1906	0.1246	-	-	-	-	0.0919
SNP_at_chr13_1078529	0.4436	0.4675	0.3012	0.5030	0.1554	0.4780	0.3369	0.2350	0.4947	0.3368	0.2743	0.4379	0.3345	0.2273	-	0.5027	0.3456
SNP_at_chr13_1762849	-	-	-	-	-	-	0.1141	-	-	0.5211	0.0784	0.3720	0.4820	0.4507	0.2596	0.1981	0.1547
SNP_at_chr13_6180831	-	-	-	-	-	-	-	-	-	0.3947	0.5069	-	-	-	-	0.1197	0.0638
SNP_at_chr14_1008800	-	-	-	-	-	-	-	-	0.4220	-	0.2155	0.1623	0.0548	0.0580	0.4131	0.0417	0.0855
SNP_at_chr14_1292382	-	-	-	-	-	-	-	-	-	-	-	0.1623	0.0278	0.2003	-	0.4885	0.0549
SNP_at_chr15_1153373	0.5152	0.5091	0.5102	0.5098	0.5085	0.5135	0.5152	0.5085	0.5106	0.5263	0.5102	0.5111	0.5070	0.5073	0.5060	0.5106	0.5112
SNP_at_chr15_2650820	0.5149	0.5091	0.1935	0.5098	0.5085	0.5135	0.5152	0.5085	0.5106	0.5263	0.4808	0.1300	0.3345	0.2817	-	0.0816	0.3824
SNP_at_chr16_5706772	0.3708	0.2227	0.3927	0.5068	0.1554	0.4908	0.5080	-	-	-	-	-	0.1311	0.1311	-	0.3608	0.2044
SNP_at_chr16_5810767	-	-	-	-	-	-	-	-	-	0.2684	0.3722	0.4763	0.3658	0.2625	0.2257	0.2837	0.1409
SNP_at_chr16_7485372	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3852	-	0.0241

Loci name	Population name																Mean
	Yunn/	Wuha	Suzh/	SMT	BSS	WTP	BDM	WHM	WKT	SSD	KNK	Viet/	Mala/	Suma/	Luzon	Maur	
SNP_at_chr18_133058	0.5172	0.5091	0.5135	0.5098	0.5085	-	0.4011	-	-	-	0.5094	-	0.1549	0.1064	-	0.4965	0.2642
SNP_at_chr19_361166	-	-	-	-	-	-	-	-	-	0.3368	-	-	-	-	0.1108	-	0.0280
SNP_at_chr2_1341108	-	-	-	0.5098	0.5085	0.3713	0.4011	-	-	-	0.5102	0.2319	0.0548	0.0278	-	0.4388	0.1909
SNP_at_chr2_4210860	-	-	-	-	-	-	-	-	-	0.5053	0.4286	-	0.0278	0.0278	-	0.0816	0.0669
SNP_at_chr2_7286866	-	-	-	-	-	0.1024	-	-	0.5071	0.4790	0.3722	0.3478	0.4969	0.4879	0.4785	0.1906	0.2164
SNP_at_chr3_5595117	0.4866	0.5033	0.4702	-	-	0.2731	0.5080	-	0.4220	0.5263	0.4971	0.1623	0.5035	0.3658	-	0.4539	0.3233
SNP_at_chr4_3522251	-	-	-	-	-	-	-	-	0.0417	0.3368	0.2743	-	0.1064	0.0548	-	0.1981	0.0633
SNP_at_chr6_1744327	-	-	-	-	-	-	-	0.3254	0.4034	0.4421	0.3265	-	0.2625	0.3658	0.4071	-	0.1583
SNP_at_chr6_9343071	-	-	-	-	-	0.0526	-	-	0.1560	0.3368	0.4971	0.0435	0.4879	0.0548	-	-	0.1018
SNP_at_chr7_1507348	-	-	-	-	-	-	-	-	-	0.5053	0.2544	0.0435	0.3940	0.3940	0.2079	0.4787	0.1424
SNP_at_chr7_1581435	-	-	-	-	-	-	-	-	-	-	-	-	0.0548	0.1549	-	0.3608	0.0357
SNP_at_chr7_9355042	0.4980	0.5065	0.5076	0.5098	0.5085	0.5135	0.5080	0.3254	0.4885	0.1895	0.1502	0.4329	-	-	-	-	0.3211
SNP_at_chr8_1019184	-	-	-	-	0.3814	0.4225	-	0.5085	0.5098	0.5053	0.5098	0.4638	0.4507	0.5008	0.4925	0.4539	0.3249
SNP_at_chr9_4822318	-	-	-	-	-	-	-	-	-	-	0.4808	-	0.3731	0.4190	0.0233	0.3369	0.1021
SNP_at_chr9_8033023	-	-	-	-	-	-	-	-	-	-	0.1502	0.1623	0.1311	0.0548	0.0681	0.4638	0.0644
Mean	0.2392	0.2555	0.2484	0.1797	0.2244	0.2403	0.2208	0.1526	0.1605	0.1757	0.1846	0.2013	0.1315	0.1204	0.0868	0.1296	0.1845
262799340	*																
262799981	*																
262801624	*																
262801649	*																
262802056	*																
262802270	*																
262802463	*																
SNP_at_chr1_1366551	*																
SNP_at_chr12_676421	*																
SNP_at_chr14_285762	*																
SNP_at_chr14_701399	*																
SNP_at_chr20_758629	*																
SNP_at_chr4_1565470	*																
SNP_at_chr4_1626735	*																
SNP_at_chr8_2101276	*																
SNP_at_chr9_555616	*																

"-" mean monomorphic loci

"*" mean the loci which gain complete genotyping profile less than 90% and were excluded in further analysis.

Genetic parameters

Based on all 80 successful loci, Average observed heterozygosity (OH), expected heterozygosity (EH), inbreeding coefficient (F_{IS}) and minor allele frequency (MAF) for each population are presented in Table 4.3. The estimated OH, EH and MAF ranged from 0.2746 to 0.5447, 0.2471 to 0.4108, and 0.18 to 0.33, respectively, among all population samples. *M. mulatta* exhibited a higher level of genetic diversity based on OH (0.4575), EH (0.3803) and MAF (0.3) than *M. fascicularis* (OH: 0.3473, EH: 0.3080, MAF: 0.23). The lowest OH, EH and MAF were found among *M. f. fascicularis* references originating in Sumatra/Indonesia (OH: 0.2746, EH: 0.2471, MAF 0.18), followed by the reference populations from Singapore/Sarawak (OH: 0.2783, EH: 0.2565, MAF 0.19) and Vietnam/Cambodia (OH: 0.2751, EH: 0.2776, MAF 0.19) (Table 4.3). All OH values were higher than EH, except those for the reference populations of *M. fascicularis* from Vietnam/Cambodia and Luzon/Zamboanga which exhibited approximately equal values of OH and EH. No populations exhibited evidence of inbreeding; most F_{IS} values were negative ranging from -0.33 (SMT, no.4) to -0.09 (Singapore/Sarawak and Suzhou/Kunming), while two *M. fascicularis* reference populations (Vietnam/Cambodia and Luzon/Zamboanga) showed positive F_{IS}, with values very close to zero (0.01) (Table 4.3).

The pairwise genetic distances between populations (pairwise F_{ST}) are provided in Table 4.4. All pairwise F_{ST} values are statistically significant at the 0.01 level of probability. The lowest pairwise F_{ST} was found among three Chinese *M. mulatta* populations, Suzhou/Kunming versus Yunnan/Myanmar (0.0218), followed by Suzhou/Kunming versus Wuhan (0.0267), then Yunnan/Myanmar versus Wuhan (0.0382). The highest genetic distances were for interspecific comparisons. The *M. fascicularis* reference population from Luzon/Zamboanga, exhibited genetic distances from *M. mulatta* populations from Myanmar (SMT), Laos (BDM) and China (Yunnan/Myanmar) of 0.7139, 0.6941 and 0.6834, respectively.

Table 4. 3 Average values of observed and expected heterozygosity (OH and EH, respectively), inbreeding coefficient (F_{IS}) and minor allele frequency (MAF). Dash line separates *M. mulatta* (upper) and *M. fascicularis* populations (lower).

Group no.	Population name	OH		EH		F_{IS}	MAF
		Polymor	All loci	Polymor	All loci		
1	Yunn/Myan	0.4482	0.2913	0.3680	0.2392	-0.23	0.28
2	Wuhan	0.4741	0.3081	0.3930	0.2555	-0.21	0.31
3	Suzh/Kunm	0.4090	0.2709	0.3750	0.2484	-0.09	0.29
4	SMT	0.5447	0.2383	0.4108	0.1797	-0.33	0.33
5	BSS	0.4971	0.2858	0.3903	0.2244	-0.28	0.32
6	WTPMH	0.3886	0.2672	0.3495	0.2403	-0.12	0.26
7	BDM	0.4409	0.2591	0.3758	0.2208	-0.18	0.29
8	WHM	0.4938	0.1852	0.4068	0.1526	-0.22	0.33
9	WKT	0.3388	0.1863	0.2918	0.1605	-0.17	0.22
10	SSD	0.4234	0.2064	0.3605	0.1757	-0.19	0.26
11	KNKTK	0.4016	0.2259	0.3283	0.1846	-0.23	0.25
12	Viet/Camb	0.2751	0.1995	0.2776	0.2013	0.01	0.19
13	Sing/Sara	0.2783	0.1426	0.2565	0.1315	-0.09	0.19
14	Suma/Indo	0.2746	0.1339	0.2471	0.1204	-0.11	0.18
15	Luzo/Zamb	0.3117	0.0857	0.3155	0.0868	0.01	0.23
16	Mauritius	0.3280	0.1476	0.2880	0.1296	-0.14	0.21
Mean *M. mulatta*		0.4575	0.2744	0.3803	0.2298	-0.21	0.30
Mean *M. fascicularis*		0.3473	0.1681	0.3080	0.1492	-0.12	0.23
Overall		0.3955	0.2146	0.3396	0.1845	-0.16	0.26

Pairwise-Fst	Yunn/ Myan	Wuhan	Suzh/ Kunm	SMT	BSS	WTP MH	BDM	WHM	WKT	SSD	KNK TK	Viet/ Camb	Sing/ Sara	Suma/ Indo	Luzo/ Zamb
Yunn/Myan															
Wuhan	0.0382														
Suzh/Kunm	0.0218	0.0267													
SMT	0.1891	0.2162	0.1904												
BSS	0.1810	0.1791	0.1501	0.2109											
WTPMH	0.0929	0.1174	0.0788	0.1780	0.1548										
BDM	0.1447	0.1284	0.1343	0.1700	0.1534	0.1395									
WHM	0.3201	0.2720	0.2564	0.3819	0.2585	0.2244	0.2948								
WKT	0.3332	0.2914	0.2844	0.3892	0.2965	0.2391	0.3158	0.2317							
SSD	0.3949	0.3608	0.3617	0.4850	0.3657	0.3248	0.3898	0.3224	0.1855						
KNKTK	0.4270	0.3938	0.4067	0.4590	0.3741	0.3798	0.3998	0.3814	0.2910	0.2394					
Viet/Camb	0.2908	0.2704	0.2427	0.3791	0.2692	0.2217	0.2819	0.2274	0.1666	0.2624	0.3171				
Sing/Sara	0.5631	0.5267	0.5383	0.6111	0.5362	0.5375	0.5687	0.5337	0.4684	0.3484	0.2289	0.4180			
Suma/Indo	0.5971	0.5598	0.5735	0.6427	0.5696	0.5736	0.6052	0.5740	0.5067	0.4000	0.2656	0.4726	0.0550		
Luzo/Zamb	0.6834	0.6455	0.6568	0.7139	0.6459	0.6622	0.6941	0.6582	0.6130	0.5488	0.4229	0.5971	0.3220	0.2909	
Mauritius	0.5928	0.5565	0.5750	0.6298	0.5650	0.5633	0.5961	0.5894	0.5433	0.4731	0.3396	0.5046	0.2898	0.2538	0.5079

Table 4. 4 Pairwise Fst values between populations of *M. mulatta* and *M. fascicularis*. Dash line separates *M. mulatta* (upper) and *M. fascicularis* populations (lower)

Genetic structure

DeltaK for STRUCTURE runs with K set from 2 to 15 showed maximum likelihood when K is 2, both based on only the admixture panel (*M. mulatta* and *M. fascicularis* populations, Figure 4.2(b)) and only the ancestry panel (within *M. fascicularis* populations, Figure 4.3(b)); therefore, the STRUCTURE plots for only K = 2 are shown here (Figure 4.2(a) and Figure 4.3(a) based on the admixture and ancestry panels, respectively). The admixture bar plot which was constructed based on 41 SNPs (Figure 4.2(a)) shows *M. mulatta* and *M. fascicularis* reference populations clearly separated from each other, with the major exception of one *M. f. fascicularis* population from Indochina (Vietnam/Cambodia), the area of the hybrid zone in which genetic admixture occurs. The proportion of genetic admixture varies across locations for both species. Thai and Laos *M. mulatta* populations from Ban Sang School (BSS, no.5), Wat Tham Pa Mak Ho (WTPMH, no.6) and Ban Dong Muang (BDM, no.7) showed some (less than 18%) *M. fascicularis* ancestry while Myanmar *M. mulatta* (Shin Ma Taung (SMT no.4)) showed almost no (less than 0.5%) *M. fascicularis* ancestry. *M. mulatta* from Thailand (BSS and WTPMH) possess more genetic admixture (17.8% and 14.3% *M. fascicularis* ancestry, respectively) than those in Laos (BDM, 5.7% *M. fascicularis* ancestry). In contrast, *M. mulatta* ancestry in *M. f. fascicularis* populations (up to 50%) exceeded *M. fascicularis* ancestry in *M. mulatta*. All populations (Wat Ham Moon (WHM, no.8), Wat Khao Thamon (WKT, no.9), Suan Somdetprasrinakharin Chumphon (SSD, no.10) and Khao Noi/Khao Tang Kuan (KN/KTK no, 11) exhibited levels of gene flow from *M. mulatta* that are consistent with their distance from the hybrid zone. WHM (no.8), the northernmost *M. f. fascicularis* population (ca. 16° 51´ N), showed the highest proportion of *M. mulatta* ancestry (50.05 %), followed by WKT, no.9 (30.09 %), SSD, no.10 (21.43 %) and KN/KTK, no.11 (15.46 %).

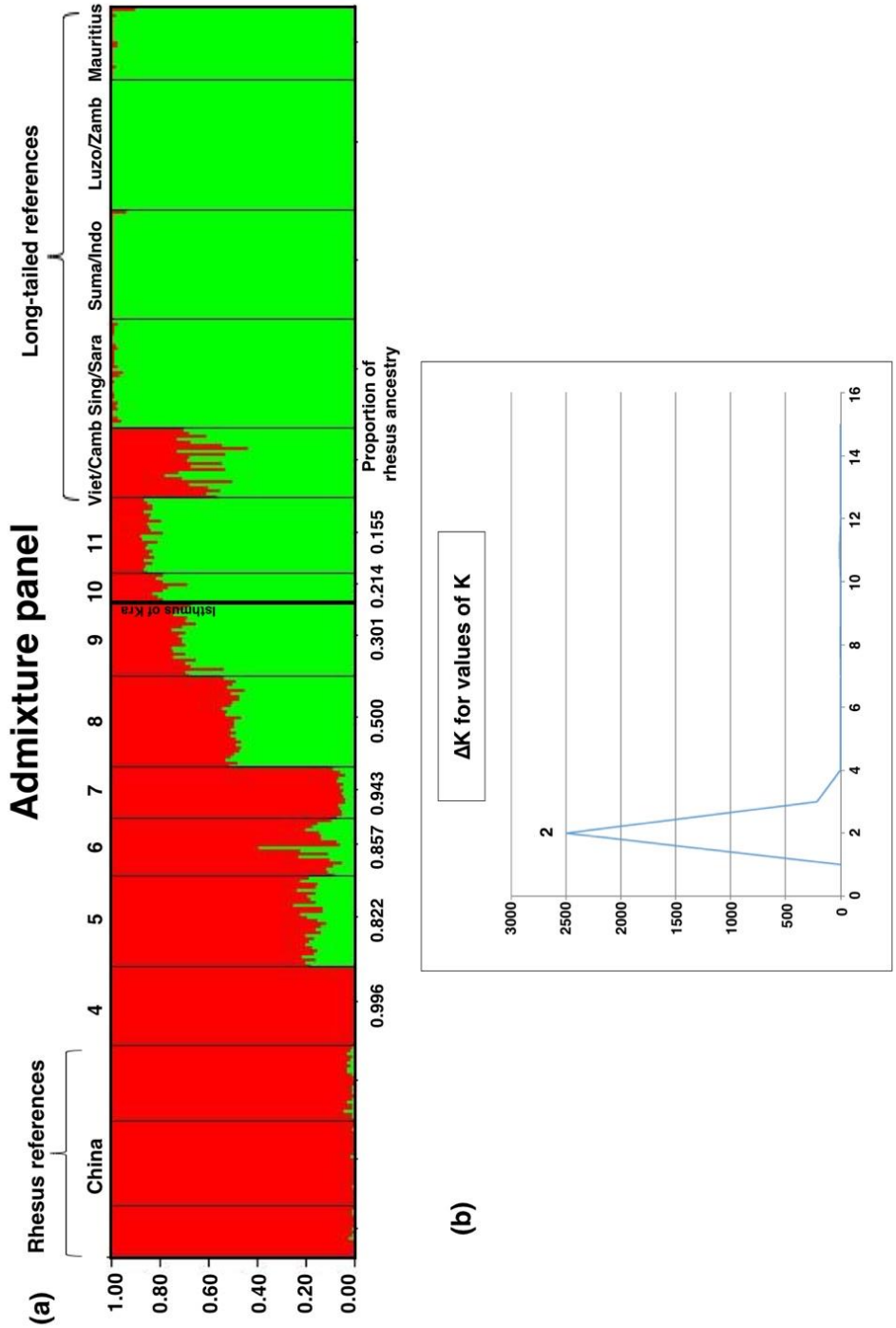

Figure 4.2 (a) Genetic structure of *M. mulatta* and *M. fascicularis* based on the admixture panel. Red and green colors indicate *M. mulatta* and *M. fascicularis* ancestry, respectively. Number 4-11 correspond to the number occurred in Table4.3 (b) deltaK for values of K in the admixture analysis.

The highest probability for the STRUCTURE ancestry analysis runs for *M. fascicularis* (Figure 4.3(a)) with K from 2 to 15 also occurred when deltaK was 2 (Figure 4.3(b)). Based on 39 successful SNPs genotyping, ancestry bar plot showed clear separation between Indochinese (Vietnam/ Cambodia) and Sunda references (Singapore/ Sarawak, Sumatra/ Indonesia, Luzon/ Zamboanga and Mauritius). The composition of WHM (no.8) and WKT (no.9) is similar to that of the Indochinese reference population whereas two other populations (SSD, no. 10 and KN/KTK, no.11) showed some level of mixed ancestry and were intermediate between those of Indochina and Sunda. The ancestry panel results showed a pattern similar to, but more distinctive than, the admixture panel results. For example, the distinction between WKT (no.9) and SSD (no.10) and between SSD (no. 10) and KN/KTK (no.11) become more pronounced.

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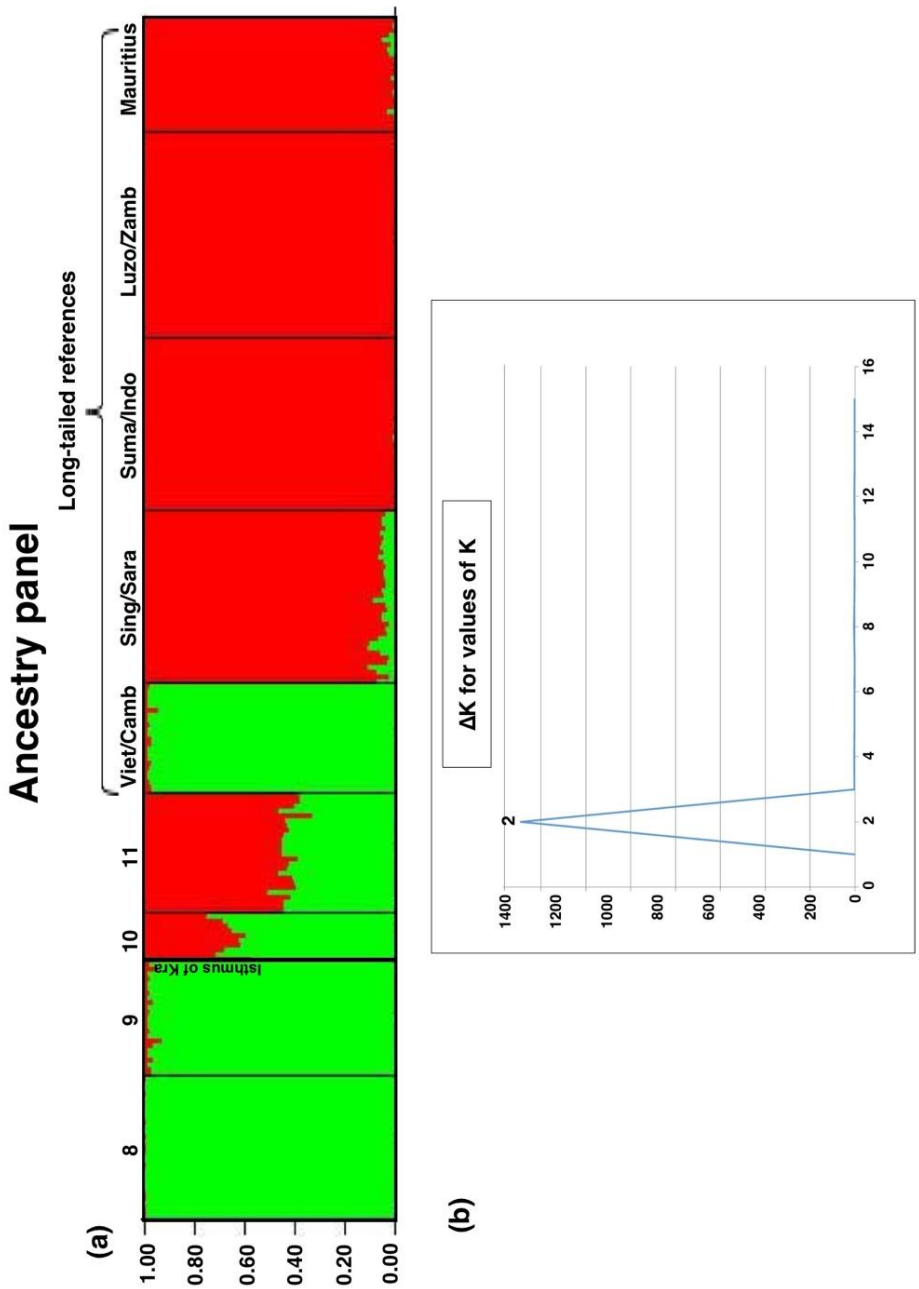

Figure 4.3 (a) Genetic structure among *M. fascicularis* populations based on the ancestry panel. Green and red colors indicate Indochinese and Sundaic ancestry, respectively. Number 8-11 correspond to the number occurred in Table 4.3 (b) deltaK for values of K in the admixture analysis.

Discussion

M. mulatta and *M. f. fascicularis* are important animal models that have been used in biomedical research since the first primate breeding station, the Soviet Institute of Experimental Pathology and Therapy (IEPT), was established in 1923 (Johnsen, Johnson, & Whitney, 2012). However, these two species live in adjacent natural habitats and have become hybridized (Fooden, 1964; Tosi et al., 2002), leading to greater complexity in their genomes. Since hybridization can be regarded as an important mechanism in the speciation process, it provides a crucial focus for evolutionary studies (Arnold & Meyer, 2006). Although many morphological and genetic studies of hybridization have been performed for more than a decade (Bonhomme et al., 2009; Hamada et al., 2008; Tosi et al., 2002), the macaque hybridization scenario and its consequence are still not fully understood, in part due to difficulties of sample collection from wild habitats in the hybrid zone. This is especially true in regard to Thailand, a country that has banned all primate exportation (Linzey & Tutu, 2013). Only recently have studies analyzing mtDNA and Y-chromosomes of *M. mulatta* and *M. f. fascicularis* throughout Thailand identified complications due to ancient and present biogeographical factors (Bunlungsup et al., 2017). However, uni-parental markers such as mtDNA and Y-chromosome – while suitable for the study of hybridization – are less powerful to detect the degree of hybridization than autosomal markers. The study of autosomal SNPs in *M. mulatta* and *M. f. fascicularis* samples from the hybrid zone and its vicinity together with reference samples provides for a more comprehensive exploration of the hybridization scenario.

Hybridization scenario

The admixture bar plot analysis supports a bi-directional hybridization scenario (Hamada et al., 2016; Kanthaswamy et al., 2010; Osada et al., 2010). Both *M. mulatta* and *M. f. fascicularis* populations living within and outside the hybrid zone have experienced

inter-specific gene flow that was biased by more extensive gene flow from *M. mulatta* to *M. f. fascicularis*. All four *M. f. fascicularis* populations in Thailand, which ranged from 16°51´ N (WHM, no.8) to 7°12´ N (KN/KTK, no.11), exhibit *M. mulatta* ancestry that declines from 50 to 15 percent over the 9 degrees of latitude. This result agrees with previous reports that the genetic introgression from *M. mulatta* to *M. f. fascicularis* population extends far beyond Indochina and the Isthmus of Kra (Bunlungsup et al., 2017; Kanthaswamy et al., 2010; Osada et al., 2010), the area previously proposed to restrict *M. mulatta* gene flow southward (Tosi et al., 2002). On the other hand, introgression from *M. fascicularis* to *M. mulatta* is more restricted, ranging from 18 percent at the BSS (no.5, 17° 51´ N) to almost zero at the northernmost population (SMT, no.4) at 21°58´ N. Although BDM (no. 7) is the southernmost *M. mulatta* population (16°54´ N), their genetic admixture with *M. fascicularis* is less than that of BSS (no. 5) and WTPMH (no. 6) which distribute at 17°51´ N and 17°14´ N, respectively. This result corresponds with the hybrid area drawn in the previous studies that demonstrates that the hybrid zone is not just a straight line, but seems to be more extensive or narrow in some areas between 15-20° N depending on the topography where the two species could be parapatric (Fooden, 1995; Fooden, 2000; Hamada et al., 2006).

Based on an autosomal analysis in this study together with the previous mtDNA/Y-chromosome analyses (Bunlungsup et al., 2017) and the practices of female philopatry and male dispersal, it can be proposed that WTPMH (no. 6) was originally an unmixed population of *M. f. fascicularis* that lived close to *M. mulatta* populations and subsequently experienced extensive genetic introgression leading to their recently reported *M. mulatta*-like morphology (Hamada et al., 2006). The hybridization scenario involving morphology, mtDNA, Y-chromosome and autosomes is illustrated in Figure 4.4. It seems likely that the genetic introgression from *M. mulatta* to *M. f. fascicularis* was maximized around the hybrid zone then declined gradually in proportion to distance from the hybrid zone and ended somewhere between the southern part of Thailand (KN/KTK, no.11) and Singapore/Sarawak. On the other hand, gene flow from

M fascicularis to *M. mulatta* was much more restricted and ended somewhere between BSS, no.5 (17°51´ N) and SMT, no.4 (21°58´ N).

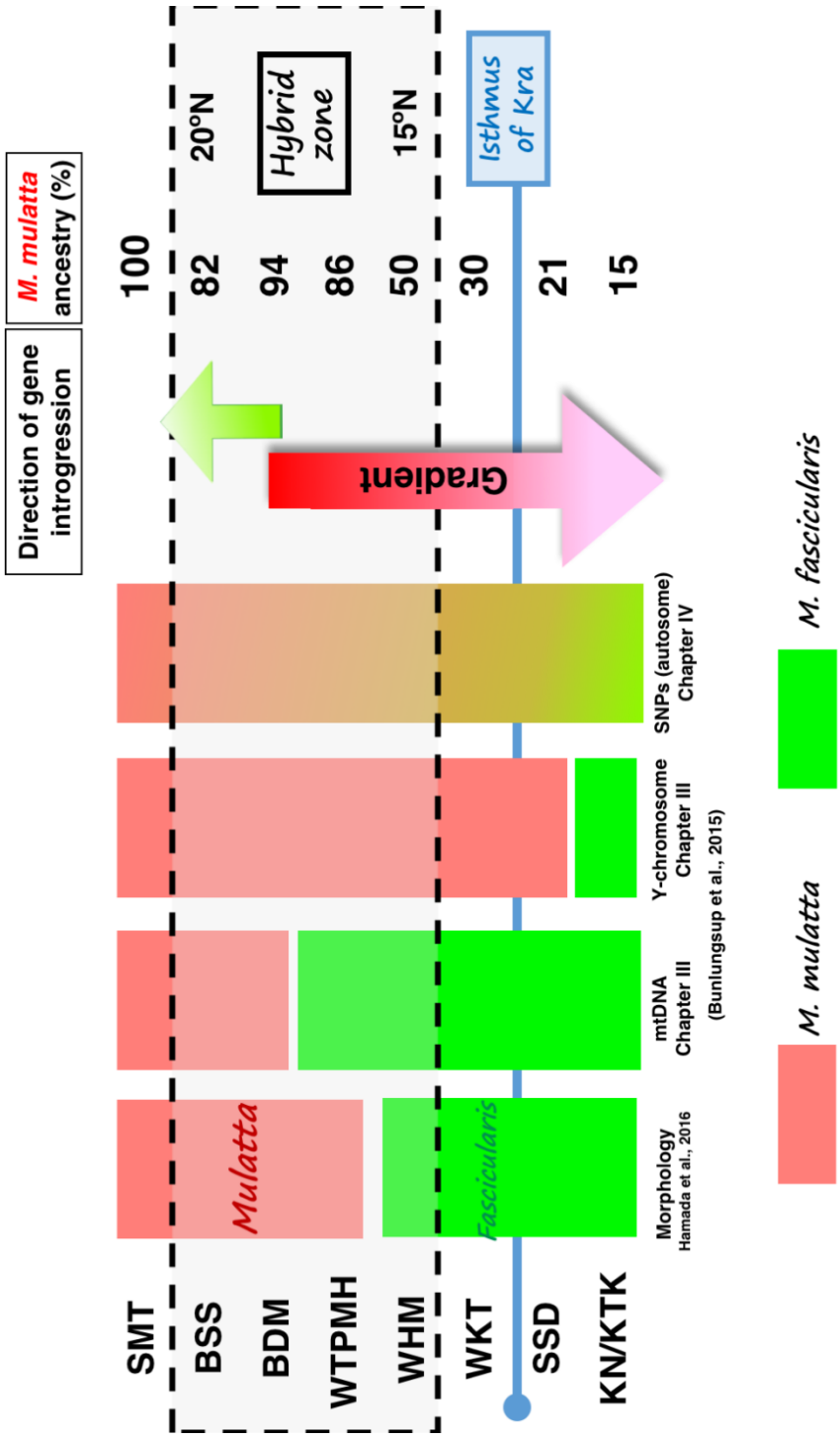

Figure 4.4 Hybridization scenario across morphological characteristic, mtDNA, Y-chromosome and autosome. Dash lines indicate the proposed hybrid area (following Fooden, 1995; Fooden, 2000; Hamada et al., 2006). Red and green colors indicate *M. mulatta* and *M. f. fascicularis* characteristics, respectively.

The very small amount of inter-species admixture which was found in Chinese *M. mulatta* and Singapore/Sarawak *M. f. fascicularis* may simply reflect a bias in marker selection. Given the considerable distance of 13 degree latitude between the *M. mulatta* populations in China, especially that from Wuhan (assumed as 30°59´ N) and BSS (17°51´ N), it seems unlikely that the low level of gene introgression of *M fascicularis* ancestry (18%) from BSS (no.5) that declined to almost zero for those at SMT (no.4) permeated northward through such a wide area in China. However, because only a few Chinese populations were analyzed in this study, the possibility that Chinese *M. mulatta* have also experienced some introgressive gene flow from *M. fascicularis* cannot be rejected. Even so, the minimal admixture of Chinese *M. mulatta* and Singapore/Sarawak *M. f. fascicularis* probably contributes little to their respective genomes.

Previous studies suggested that the hybridization between these two species was caused by climate fluctuations that fostered migrations during glacial and interglacial episodes of the Pleistocene, a million or more years ago (Bonhomme et al., 2009; Osada et al., 2010). *M. mulatta* that lived in northern latitudes may have migrated southward to access the warmer temperatures during glacial advances while *M. f. fascicularis* spread northward during interglacial episodes leading to hybridization. At present, deforestation resulting in habitat loss and forest fragmentation might have limited the extent of genetic introgression among macaque populations.

Thus, the asymmetry of hybridization between *M. mulatta* and *M. f. fascicularis* could be explained by three reasons. First, because *M. mulatta* are larger and heavier in size than *M. f. fascicularis* (Hamada et al., 2006), either *M. mulatta* male coerces *M. f. fascicularis* female to mate with them or *M. f. fascicularis* female prefers *M. mulatta* male because they are stronger and/or more aggressive than their conspecific males. The difference in reproductive patterns of the two species may constitute an additional influence on asymmetry of introgression. While *M. mulatta* give birth seasonally (Weinbauer et al., 2008), *M. f. fascicularis* can give birth throughout the year with a peak

during May–July or a few months later depending on food availability (Kavanagh & Laursen, 1984; van Schaik & Noordwijk, 1985). Consequently, *M. mulatta* females are generally less frequently receptive to mating with either conspecific or non-conspecific males compared to *M. f. fascicularis* females. Additionally, the ovulatory signals sent from *M. mulatta* and *M. f. fascicularis* females to their conspecific males exhibit different patterns. *M. mulatta* females exhibit sex skin reddening covering the whole areas surrounding the ischial callosities down to the thigh, while the sex skin swelling of *M. f. fascicularis* females is restricted at the base of the tail (Engelhardt et al., 2005, Jadejaroen et al., 2015). Taken together, these two factors provide a longer time for *M. mulatta* male to learn visual cues of ovulation in *M. f. fascicularis* female and mate with them.

Genetic differentiation among *M. fascicularis fascicularis*

Two factors contributed to genetic differentiation between Indochinese and Sundaic *M. f. fascicularis*. First, the genetic invasion of *M. mulatta* southward profoundly influenced the genome of Indochinese, but not Sundaic, *M. f. fascicularis*. Second, the Isthmus of Kra (10° 30´ N) and Kangar-Pattani line (6° 30´ N) in the southern part of Thailand established a biogeographical barrier that divided Indochina and Sunda into two different zoogeographic sub-regions in the Southeast Asia continent (Wallace, 1876). Many studies have distinguished assemblages of animals and plants between these two landmarks (Denduangboripant & Cronk, 2000; Hughes et al., 2011; Hughes et al., 2003; Malaivijitnond et al., 2012). Although the geographic distances among the Thai populations WHM, WKT, SSD and KN/KTK were similar (approximately 2.5-3.5 degree latitude), genetic differentiation between WHM and WKT was minimal while that between WKT-SSD and SSD-KN/KTK was much greater. This is because WHM and WKT are Indochinese populations that experienced similar levels of genetic introgression from *M. mulatta* due to the absence of intervening geographic barriers.

The Isthmus of Kra (~10° 30´ N), which lies between WKT (no.9) and SSD (no.10), is one of the most well-known zoogeographical barriers proposed as the boundary between northern (Indochina) and southern (Sunda) animal distributions (Bunsong & MacNeely, 1988; Hughes et al., 2003). The Kangar-Pattani line (~6° 30´ N), located between the borders of Thailand and Malaysia, near the KN/KTK population (~7° 12´ N), is well known among botanist as the major Indochinese-Sundaic plant boundary separating the perhumid evergreen rainforest from the wet seasonal evergreen rainforest (Steenis, 1950). Wallace (1876) regarded the Kangar-Pattani line as the boundary between these two regions, a proposal that was later supported by many other studies (Hughes et al., 2011; Woodruff & Turner, 2009). Tougard (2001) hypothesized that in the Pleistocene epoch, when the northern hemisphere was cooler than at present, the boundary between Indochina and Sunda may have shifted due to climate changes that precipitated animal migration. In addition, Malaivijitnond et al. (2012) found the separation between southern and northern pig-tailed macaques to be the Surat thani-Krabi depression (~8-9° 30´ N), the area between Isthmus of Kra and Kangar-Pattani line. Thus, at least three geographic barriers among macaque species have been recognized: the Isthmus of Kra, the Surat thani-Krabi depression and the Kangar-Pattani line. The relative effectiveness of these three locations as barriers to inter-species gene flow undoubtedly varied over time. Based on the impact of these biogeographical barriers, it is not surprising that Indochinese monkeys exhibit low levels of genetic differentiation while the monkeys of southern Thailand, the area recognized as the transition zone between the two regions, exhibit dramatic genetic differentiation.

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CHAPTER V
MORPHOLOGICAL CHARACTERISTICS AND GENETIC DIVERSITY OF *Macaca fascicularis aurea*

Introduction

Macaca fascicularis are the second most widely distributed and diversified macaque (after *M. mulatta*), being found in a geographic area that encompasses continental and insular populations, which lead to their high genetic diversity (Fooden, 1995). They have been classified into 10 subspecies based on their different geographic origins and morphological characteristics (Fooden, 1995). A large number of studies focused on *M. f. fascicularis* is attributable to the much more widespread distribution of that subspecies than all others (Abdul-Latiff et al., 2014b; Blancher et al., 2008; Li et al., 2012; Kanthaswamy et al., 2013; Tosi & Coke, 2007). *M. f. fascicularis* distributes throughout mainland and many islands of Southeast Asia which leads to their common name of common long-tailed macaques. Moreover, they are popularly used as a primate model in the biomedical research. For *M. f. aurea*, they are listed as data deficient for International Union for Conservation of Nature (IUCN). This subspecies is strikingly important because it is the only one of the three non-human primate species that has been reported to use stone-tools to access protected food items, such as oysters (Gumert et al., 2009, 2011, 2013; Gumert & Malaivijitnond, 2012, 2013; Malaivijitnond et al., 2007). *M. f. aurea* lives mainly in Myanmar, which leads to their common name of Burmese long-tailed macaques, and is distributed southeastward along the Andaman seacoast through the Mergui Archipelago and western Thailand (Fooden, 1995; Malaivijitnond & Hamada, 2008; San & Hamada, 2011). The major threats to Burmese subspecies is habitat fragmentation, encroachment of it mangrove forest habitats for shrimp culture and agriculture practices, hunting for food and trade (Gumert et al., 2013; Kabir & Ahsan, 2012; San & Hamada, 2011).

Fooden (1995) noted that the key morphological character that can be used to differentiate *M. f. fascicularis* and *M. f. aurea* is the lateral facial crest pattern. *M. f. fascicularis* has a transzygomatic crest hair pattern, where the crest sweeps upward from near the angle of the jaw to the lateral margin of the crown, passing over the zygomatic bone locating between the eye and ear. In contrast, *M. f. aurea* has an infrazygomatic facial crest pattern, where the hairs of the temporal region are smoothly directed posteriorly from the posterior margin of the eye to the anterior margin of the ear, and sometimes the hairs form a whorl, below the zygomatic bone. Generally, the morphological characters of *M. f. aurea* are similar to those of *M. f. fascicularis*, except that *M. f. aurea* look darker, especially on the face and nose (Fooden, 1995).

Regarding to their distribution ranges, Burmese subspecies lives in close contact with *M. mulatta* in central Myanmar and with common subspecies in southwestern Thailand at the vicinity of the Isthmus of Kra (approximately 10° 30′ N) Hybrids between the different species (*M. f. aurea* × *M. mulatta*) or different subspecies (*M. f. aurea* × *M. f. fascicularis*) have been reported or proposed (Fooden, 1995; Hamada et al., 2006).

With respect to the Isthmus of Kra zoogeographical barrier, *M. f. fascicularis* from the north and south of the Isthmus of Kra were morphologically (Hamada et al., 2008) and genetically different (Tosi et al., 2002), and separated into two major forms (Indochinese and Sundaic). Basically, the latter had a longer tail and smaller contrast of the yellow pelage color between the back and the thigh (Hamada et al., 2008). One possible reason to explain these differences is that the *M. f. fascicularis* inhabiting north of the Isthmus of Kra are hybrids derived from the introgression of *M. mulatta* males (Bonhomme et al., 2009; Kanthaswamy et al., 2008, 2010; Osada et al., 2010; Satkoski, et al., 2013; Stevison & Kohn, 2009; Tosi et al., 2002; Yan et al., 2011) while the southern population do not gain such a genetic introgression much from *M. mulatta* compared with the northern population (Chapter III and IV). On contrarily to the well-

known study of the hybridization between *M. mulatta* and *M. f. fascicularis*, no genetic studies on the hybridization between *M. f. aurea* and *M. f. fascicularis* have been conducted in last 20 years after the intersubspecific contact zone was proposed (Fooden, 1995). Only one genetic study of *M. f. aurea* was recently carried out using microsatellite markers, but those monkeys were originated in Cambodia which is not within the habitat range of *M. f. aurea* (Li et al., 2012).

Interestingly, although *M. f. fascicularis* occurs parapatrically with *M. f. aurea* and can be seen in the same habitat types, tool-use behavior has never been reported in *M. f. fascicularis* including in a 25-year field survey (Malaivijitnond & Hamada, 2008; Malaivijitnond, Vazquez, & Hamada, 2011). So far, up to five geographic populations of *M. fascicularis* have been recorded to use tools; one each at the islands in the Mergui Archipelago of southern Myanmar (Carpenter, 1887), the Piak Nam Yai Island, Baan Koh Lao Island, Pracharatrangsarith Temple (Gumert et al., 2009; Malaivijitnond et al., 2007) and the Koram Island of Thailand (Aiempichitkijkarn et al., 2014). All these populations were comprised of the *M. f. aurea* except at Koram Island (KRI; the only population in the Thai Gulf), where the monkeys appeared by cheek hair pattern to be hybrids between *M. f. aurea* and *M. f. fascicularis*. However, these tool using macaques all lived in similar habitat types of islands or fringes of mangrove forests with encased marine invertebrate foods and the availability of stone tools. There has been no report of tool use behavior in other mainland dwelling *M. f. aurea* (San & Hamada, 2011).

Thus, the genetic study of *M. f. aurea*, *M. f. fascicularis* and their hybrids from the wide origins in this study could shed light on *M. f. aurea* and *M. f. fascicularis* evolutionary history including potentially the relationship between genetics and their tool use behavior which is unique only in *M. f. aurea*.

Methods

Sample collection and DNA extraction

Blood or fecal samples of free ranging *M. f. aurea*, *M. f. fascicularis*, and hybrids from eight, five, and four populations, respectively, were analyzed (Table 5.1 and Figure 5.1). The eight populations of *M. f. aurea* covered their distribution range, except in southern Bangladesh (Fooden, 1995), where it has previously reported as possibly no longer exist (Kabir & Ahsan, 2012). Five populations of *M. f. fascicularis* included in this study lived parapatrically with *M. f. aurea* both in the Indochina and Sunda regions, where the four populations of morphological hybrids were attained. In addition, *M. mulatta* derived from China and reared at the Primate Research Institute of Kyoto University, Japan, was included in the analysis. Monkeys were identified the species, subspecies or hybrids with regard to their morphological characters, mainly on their cheek hair pattern, vertex of head crest, and pelage color (Fooden, 1995; Hamada et al., 2006, 2008).

All DNA samples extracted from blood or fecal samples were derived from DNA Bank of the Primate Research Unit, Chulalongkorn University (Malaivijitnond & Hamada 2008, Malaivijitnond et al., 2008). The experimental protocol was approved by the Institutional Animal Care and Use Committee of Faculty of Science in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University, Protocol Review No. 1423010.

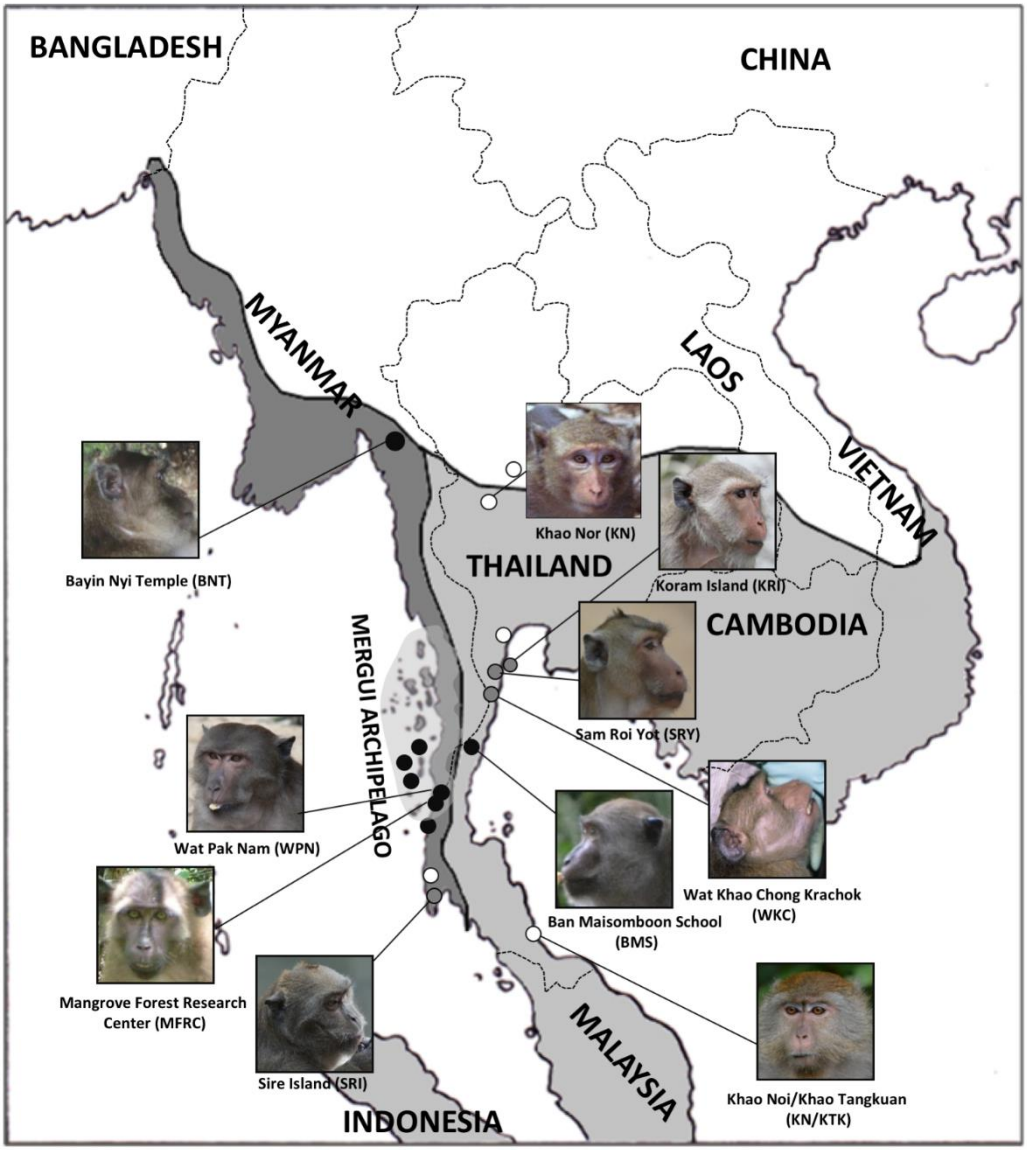

Figure 5. 1 Distribution range of *M. f. aurea* (Burmese subspecies; black) and *M. f. fascicularis* (common subspecies; gray). Black, white, and gray circles indicate the Burmese subspecies, common subspecies, and hybrid locations of the samples collected in this study.

Table 5. 1 Region, locality, geographical coordinate, morphological species identification, collected specimen and the observation of tool use in *M. f. fascicularis*, *M. f. aurea* and the hybrid between the two species. Bold letter indicates the hybrid. The species was identified based on the morphological characters.

Region	Name of Location	GPS (North, East)	Taxon	Specimen	Observation of tool use
Thailand (Indochina)	1. Wat Haad Moon Bang Kra Beau (WHM)	16º 30′, 100º 16′	*M. f. fascicularis*	Blood	No
	2. Wat Khao Nor (KN)	15º 57′, 99º 52′	*M. f. fascicularis*	Blood	No
	3. Wat Khao Thamon (WKT)	13º 02′, 99º 57′	*M. f. fascicularis*	Blood	No
	4. Koram Island (KRI)	**12º 14′, 100º 00′**	**Mixed**	**Feces**	**Yes**
	5. Samroiyot National Park (SRY)	**12º 07′, 99º 57′**	**Mixed**	**Feces**	**No**
	6. Wat Khao Chong Krachok (WKC)	**11º 48′, 99º 48′**	**Mixed**	**Blood**	**No**
	7. Banmaisomboon School (BMS)	10º 51′, 99º 13′	*M. f. aurea*	Feces	No
Thailand (Sunda)	8. Wat Paknam Pracharangsarith (WPN)	9° 57′, 98° 35′	*M. f. aurea*	Blood	Yes
	9. Mangrove Forest Research Center (MFRC)	9º 52′, 98º 36′	*M. f. aurea*	Blood	No
	10. Piak Nam Yai Island (PNY)	9º 35′, 98º 28′	*M. f. aurea*	Feces	Yes
	11. Wat Suwan Khuha (WSK)	8º 25′, 98º 28′	*M. f. fascicularis*	Blood	No
	12. Sirae Island (SRI)	**7º 54′, 161º 98′**	**Mixed**	**Feces**	**No**
	13. Khao Noi/Khao Tangkuan (KN/KTK)	7º 12′, 100º 35′	*M. f. fascicularis*	Blood	No
Myanmar	14. Bayin Nyi Temple (BNT)	16º 58′, 97º 29′	*M. f. aurea*	Blood	No
	15. Lampi Island (LPI)	10º 54′, 98º 12′	*M. f. aurea*	Feces	Yes
	16. Jarlan Island (Lord Loughbrough) (JLI)	10º 25′, 97º 56′	*M. f. aurea*	Feces	Yes
	17. Zadetkyi (ZDK)[a]	9º 58′, 98º 11′	*M. f. aurea*	Feces	Unknown*
China	18. Reared at the Primate Research Institute of Kyoto University, Japan	Unknown	*M. mulatta*	Blood	No

Subspecies were identified based on their morphological characters (Fooden, 1995; Hamada et al., 2008).

[a]This specimen was a pet macaque, which had been captured from Zadetkyi.

mtDNA and Y-chromosome gene amplification and sequencing

To trace the genetic differentiation among populations, an approximately 835 bp mtDNA fragment including the HVSI of the D-loop region, tRNA proline, tRNA threonine, and cytochrome b was PCR amplified. The PCR amplification was performed following Smith & McDonough (2005) using the HVS-F/R primer pair. Note that the HVS-F/R primers were designed to avoid the nuclear–mitochondrial insertion (numt) region, and if the ambiguous sequences were found, the cloning was performed.

To trace the paternal inheritance, migration, and introgression pattern of the males, the sex-determining region Y-chromosome (*SRY*) and testis-specific protein Y-chromosome (*TSPY*) genes, which are approximately 800 bp and 2.3 kbp in length, respectively, were amplified. These two loci are located on the non-recombinant portion and mapped onto the short and long-arm of Y-chromosome, respectively. The *SRY* gene was amplified using the SW2/SW3B primer pair as described by Whitfield et al. (1993) except with a slight modification to the annealing temperature (see below). The *TSPY* gene was amplified from the blood DNA samples using the TSPY-A/TSPY-5R primer pair following Tosi et al. (2000). However, due to the degradation of the fecal DNA samples, three pairs of primers (TSPYA/ TSR1012, TSF566/ TSR1676, and TSF1383/TSPY-5R for 1012, 1110, and 855 bp of sequence, respectively) were used for amplification to cover the whole length of targeted *TSPY* gene as described previously in Chapter III (Bunlungsup et al., 2015).

PCR mixtures (10 µl total) contained 0.625U ExTaq DNA Polymerase (Takara Bio Inc., Shiga, Japan), 0.2 mM each primer and 50–100 ng DNA template in the manufacturer's buffer. In addition, for the extracted DNA from fecal samples, 0.12 mg T4 gene 32 protein (Wako Nippon Gene, Japan) was included to promote the DNA synthesis by DNA polymerase. The mtDNA amplification was performed with 40 cycles of 94°C for 25 sec, 63 to 60°C (decreasing at –0.1°C/cycle) for 30 sec and 72°C for 20 sec, and then followed by 72°C for 7min. The *SRY* gene amplification was

performed with 40 cycles of 94°C for 30 sec, 52–57°C for 20 sec, and 72°C for 2 min, followed by 72°C for 7min. The *TSPY* gene amplification from blood DNA samples was performed with 45 cycles of 94°C for 25 sec, 66°C for 45 sec, and 72 °C for 3min, followed by a final 72°C for 7 min. Due to the long length of the *TSPY* gene, the amplification from fecal DNA was performed with three pairs of primers and various annealing temperatures with a reduced extension time to 1 min.

All PCR reaction products were resolved and visualized on 1% (w/v) agarose gel-TAE electrophoresis followed by ethidium bromide staining and UV-transillumination. Specific PCR products were directly purified using the ExoSAP-IT kit (Affymetrix Inc., CA). Sequencing reactions were performed on both strands using the same PCR primers (individually), except the *TSPY* gene long amplicon acquired from the blood DNA was sequenced additionally with two addition primers pairs as described in Chapter III. Sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3130xL Genetic Analyzer (Applied Biosystems, CA) . Data output was assembled and analyzed on SeqMan^{tm} II (DNASTAR Inc.) and Finch TV (Geospiza Inc., WA).

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Data analysis and phylogenetic tree construction

Although *SRY* and *TSPY* showed similar topology, the partition homogeneity was tested by PAUP 4.0 program with 1,000 bootstrap (P=1.0, Swofford, 2003). Since such a test showed significant homogeneity signal (P=1.0), the data were combined (concatenated) to one sequence containing 2,040 bp of *TSPY* and 686 bp of *SRY* for phylogenetic analysis (Tosi et al., 2000, 2002; Tosi & Coke, 2007). The corresponding DNA sequences from *M. sylvanus* (Barbary macaque) were downloaded from Genbank and used as the outgroup (Accession numbers: NC_002764 for mtDNA, and AF284326, and AF284275 for the *SRY* and *TSPY* genes, respectively). The *SRY* and *TSPY* sequences of the Sundaic common subspecies from Indonesia (Java) and Malaysia (Selangor and

Johor) (Accession numbers: AF425293, AF425294, AF425292, and AF284303 for *SRY* and AF425278, AF425279, AF425277, and AF284252 for *TSPY*) were also downloaded and included in the analysis to improve the resolution of any hybridization between Burmese subspecies and common subspecies. All DNA sequences were aligned by ClustalW as implemented in the Mega5.2 software (Tamura et al., 2011). To include all sequences into the phylogenetic analysis, both ends were trimmed off before the analysis was performed. Thus, the total of 677 and 2,726 bp of mtDNA and Y-chromosome gene, respectively, were used for analysis. The phylogenetic trees were constructed using distance-base method (Neighbor Joining) implemented in the Mega5.2 software (Tamura et al., 2011) with 1,000 bootstrap. DNA sequences were checked for any indels using Mega 5.2 software.

Result

Genetic divergence of *M. f. aurea* and *M. f. fascicularis*

Totally 55 and 39 monkeys were sequenced for mtDNA and Y-chromosome (Accession number: LC093173-LC093227 for mtDNA, LC093268- LC093306 for *SRY* gene, and LC093307- LC093345 for *TSPY* gene), respectively, and because four sequences of *SRY* and *TSPY* gene were downloaded from Genbank, in total of 43 sequences of Y-chromosome were analyzed. From the mtDNA sequence data, 155 bp were variable sites (22.9%) of which 142 sites were parsimony informative characters (21%), including 123 sites of two variants (18%), 10 sites of three variants (1.5%), and two sites of four variants (0.3%), and 9 sites were indels (5.8%). The numbers of transition and transversion were 127 and 7, respectively, and its ratio was 18.1:1. The variable sites in Y-chromosome (*SRY* and *TSPY*), however, were much fewer in comparison with that of the mtDNA, only 21 variable sites were found and 18 were informative parsimony (0.7%). Two indels were found of which they are a single-base indel in *TSPY* and a three-base indel in *SRY*. The numbers of transition and transversion of the Y-chromosome

genes were 13 and 6, respectively, and its ratio was 2.17:1.

Based on the NJ phylogenetic analysis of 677 bp of mtDNA sequence, the monkeys were divided into the two major clades of *M. f. aurea* and *M. mulatta*/*M. f. fascicularis* (Figure 5.2). Within the *M. f. aurea* clade, the population of mainland Myanmar (BNT) was separated from the populations at the Mergui Archipelago (LPI, ZDK, and JLI) and those that originated from the Thailand Andaman seacoast (PNY and MFRC). The WPN population living on the island between Myanmar and Thailand either clustered with the Mergui populations (WPN1297) or formed a separate subclade with the Thailand Andaman seacoast populations (WPN1295 and 1298). Within the *M. mulatta*/*M. f. fascicularis* clade, *M. mulatta* formed monophyletic clade and separated from *M. f. fascicularis* which was divided into the Indochinese (WHM, KN, and WKT) and Sundaic (KN/KTK and WSK) groups. All the morphological hybrids (SRY, KRI, WKC, and SRI) clustered with the *M. f. fascicularis* clade. Interestingly, the BMS population, which was identified as *M. f. aurea* by their morphological appearance, was in contrast placed within the Indochinese *M. f. fascicularis* clade.

The NJ analysis of the combined 686 and 2,040 bp *SRY* and *TSPY* sequences revealed that the monkeys were divided into the three major clades of *M. mulatta* /Indochinese *M. f. fascicularis*, Sundaic *M. f. fascicularis*, and *M. f. aurea*, respectively (Figure 5.3). The Sundaic *M. f. fascicularis* clade consisted of the Java monkeys, peninsular Malaysia (Selangor and Johor), and Sundaic Thailand (KN/KTK and WSK). The *M. f. aurea* clade was divided into three subclades of (i) monkeys from mainland Myanmar (BNT), (ii) the population living between Myanmar and Thailand (WPN), and (iii) island monkeys from the Mergui Archipelago (LPI and ZDK), Thailand Andaman seacoast (PNY) and mainland mangrove forest of Thailand (MFRC). Interestingly, the morphologically identified *M. f. aurea* with the mtDNA haplotype of *M. f. fascicularis* (BMS) were grouped with the WPN population. The *M. mulatta*/ Indochinese *M. f. fascicularis* clade had only one haplotype. Three populations of mixed morphological

characters identified as subspecies hybrids (KRI, SRY, and WKC) had Y-chromosome gene sequences grouped with either the *M. mulatta*/Indochinese *M. f. fascicularis* or *M. f. aurea* clades. Unfortunately, the fecal specimens of hybrid males from the SRI population that lived close to the southernmost group of *M. f. aurea* in the Sundaic region of Thailand could not be collected. Thus, whether the SRI population grouped with the Sundaic *M. f. fascicularis* or *M. f. aurea* is still unresolved.

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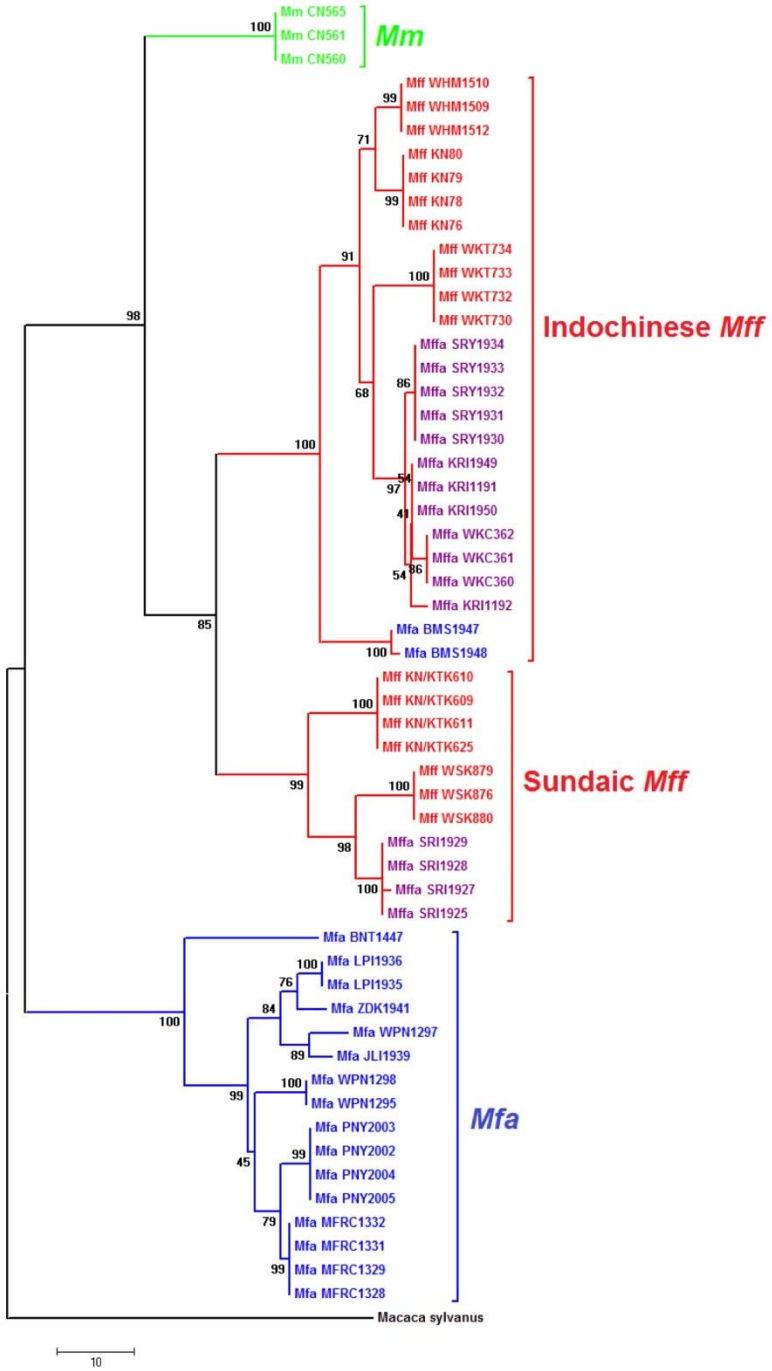

Figure 5. 2 NJ-based phylogenetic tree based on 677 bp of mtDNA. *Mm*, *Mff*, *Mfa*, and *Mffa* stand for *M. mulatta*, *M. f. fascicularis*, *M. f. aurea*, and hybrid between *M. f. fascicularis* and *M. f. aurea*, respectively. Green, red, blue, and purple letters indicate *M. mulatta*, *M. f. fascicularis*, *M. f. aurea*, and hybrid between *M. f. fascicularis* and *M. f. aurea*, respectively. The number on each branch indicates bootstrap value.

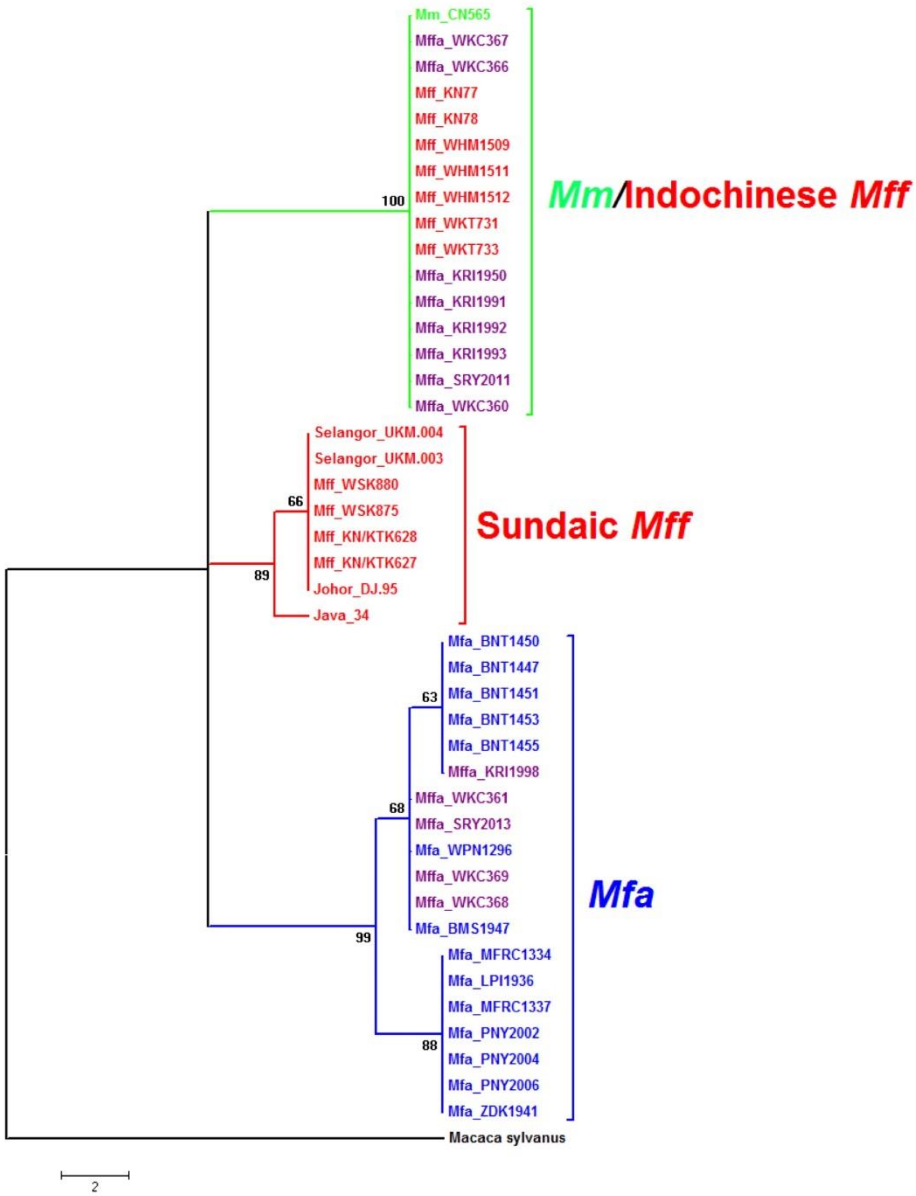

Figure 5.3 NJ-based phylogenetic tree based on the 2,726 bp of the combined *SRY* and *TSPY* gene sequences. *Mm*, *Mff*, *Mfa*, and *Mffa* stand for *M. mulatta*, *M. f. fascicularis*, *M. f. aurea*, and hybrid between *M. f. fascicularis* and *M. f. aurea*, respectively. Green, red, blue, and purple letters indicate *M. mulatta*, *M. f. fascicularis*, *M. f. aurea*, and hybrid between *M. f. fascicularis* and *M. f. aurea*, respectively. The number on each branch indicates bootstrap value.

Discussion

Based on the survey in Thailand (Malaivijitnond & Hamada, 2008a; Malaivijitnond et al., 2008b) combined with the published reports on the distribution of *M. f. aurea* in Myanmar and Bangladesh, the geographic range of *M. f. aurea* can be described as extending from the northernmost populations at the coastal forest belt along the Naf River at Keruntoli (20° 54′ N, 92° 16′ E), near the Teknaf Port in Bangladesh (Kabir & Ahsan, 2012), and then eastward to central and southern Myanmar in Rakhine, Ayeyarwady Delta, Bago Yoma, and Tanintharyi biogeographic regions (San & Hamada, 2011), and then southward along the Andaman seacoast to southwestern Thailand at PNY Island, Ranong Province (9° 35′ N, 98° 28′ E, this study). Note that PNY is the place where stone tool use behavior in *M. f. aurea* was first discovered in Thailand (Malaivijitnond et al., 2007) after the 120-year-old report of Carpenter (1887) in the Mergui Archipelago. Indeed, the PNY population was found in 2005, the year after the Tsunami impact on the Andaman coast of Thailand. Thereafter, as reported herein, searching for tool use populations along the Andaman seacoast and Mergui Archipelago has revealed three more locations of tool using macaques (WPN, LPI, and JLI).

From more than 100 populations of *M. f. fascicularis* surveyed in Thailand to date (Malaivijitnond & Hamada, 2008; Malaivijitnond et al., 2011), tool use has not been observed. In addition, no report on tool use in 22 other macaque species has been found (Groves, 2001; Mittermeier, 2013). The genetics might play an important role in the control of tool use behavior in macaques. Some support for a genetic role comes from the fact that one subspecies hybrid population (KRI), with respect to their morphological appearance and mtDNA and Y-chromosome gene analyses, was observed using stone tools. However, all the tool using macaques lived in broadly similar habitat types that consisted of encased foods, stone tools, and anvil or substrate stones. Thus, the habitat conditions could likely promote tool use behavior (necessity for acquiring food and the

local availability of suitable tools) to emerge and then the behavior could propagate within and between other nearby populations. Accordingly, the natural environment is likely to be one of the driving forces for them to learn to use tools. This is also true for the isolated islands along the Mergui Archipelago and Andaman seacoast.

The mtDNA and Y-chromosome gene sequence analyses in this study supports previous studies that Indochinese *M. f. fascicularis* had a genetic admixture from the introgression of *M. mulatta* male (Bonhomme et al., 2009; Kanthaswamy et al., 2008; Stevison & Kohn, 2009; Tosi et al., 2002; Yan et al., 2011). However, in this study using the *SRY* and *TSPY* gene sequences, the genetic admixture of *M. mulatta* was only detected in the Indochinese *M. f. fascicularis* living in Thailand (WHM, KN, and WKY populations), but not in the Indochinese *M. f. aurea* living in Myanmar (BNT population). Why hybrids between *M. mulatta* and *M. f. aurea* were not detected needs to be investigated further. Previous studies have proposed that hybrids occurred beyond the hybrid zone of 15–20° N and that the Isthmus of Kra (approximately 10° 30′ N) is the zoogeographical barrier in relation to changes in the sea level (Hamada et al., 2008; Kanthaswamy et al., 2010; Osada et al., 2010; Satkoski, et al., 2013; Tosi et al., 2000, 2002). However, verification on this point has not been ascertained, and no macaque populations in these areas have been included for DNA analyses. This might be because it is difficult to acquire the information of free ranging *M. fascicularis* in these areas and to access those populations due to the political problem in southern Thailand. In this mtDNA-based analysis, the *M. f. fascicularis* inhabiting 16° 30′–13° 02′ N (WHM, KN, and WKT) and the morphological subspecies hybrids inhabiting 12° 14′–11° 48′ N (KRI, SRY, and WKC) were grouped together and separated from the Chinese *M. mulatta* (unknown origin) and Sundaic *M. f. fascicularis* (8° 25′ to 7° 12′ N; WSK and KN/KTK). From the *SRY* and *TSPY* sequence analysis, all three populations of Indochinese *M. f. fascicularis* (WHM, KN, and WKT; 16° 30′–13° 02′ N) and the subspecies hybrids living in the Indochina region (KRI, SRY, and WKC; 12° 14′–11° 48′ N) were grouped

with the Chinese *M. mulatta*, but were separated from the peninsular (KN/KTK, WSK, Selangor, and Johor) and insular (Java) Sundaic *M. f. fascicularis* (8° 25′ N to approximately 7° S) populations.

The mtDNA sequence analysis indicated that the oldest *M. f. aurea* haplotypes were from the mainland Myanmar (BNT), which supports the previous hypothesis that *M. f. aurea* arose from a refugia population in the north of Myanmar (San & Hamada, 2011). Considering the current geographic distribution of *M. f. aurea* along with the mtDNA and Y-chromosome gene sequence analyses, this leads to the conclusion that *M. f. aurea* originated in Myanmar and migrated southward along the Mergui Archipelago through the Andaman seacoast toward southwestern Thailand when the sea level was low and the Sunda shelf was exposed. Due to the fact that, Mergui archipelago and mainland Myanmar was separated by the shallowing-water which thus, had connected many times during the Pleistocene epoch. The most recent connection between Mergui archipelago and mainland Myanmar occurred in the late-Pleistocene epoch of 21,000–9,000 years ago when the sea was lower than the present-day level up to 120 meters (Sathiamurthy & Voris, 2006).

That the hybrids could be discriminated into two groups (Indochinese and Sundaic) agrees with the two mtDNA haplotypes of Indochinese (SRY, KRI, WKC, and BMS) and Sundaic (SRI) populations of subspecies hybrids. This then denotes two possible hybridization events, namely with Indochinese or Sundaic *M. f. fascicularis*. In the first event, *M. f. aurea* migrated along the Mergui Archipelago and Andaman seacoast, where *M. f. aurea* male introgressed into Sundaic *M. f. fascicularis* populations, represented now by the SRI subspecies hybrids. Although *M. fascicularis* prefers low elevation habitats (Fooden, 1995), when the sea level was low, *M. f. aurea* male subspecies living at the Andaman seacoast migrated east-northward across the low altitude area of the Dawna range (San & Hamada, 2011) to mainland Thailand and the islands on the Thai Gulf, represented now by the SRY, KRI, and WKC hybrid

populations. The second hybridization event occurred recently because the males in these three populations still carried the Y-chromosome gene of either Indochinese *M. f. fascicularis* or *M. f. aurea*.

That the hybrids have two haplotypes of the Y-chromosome (*M. mulatta*/*M. f. fascicularis* and *M. f. aurea*) can be explained by two scenarios of hybridization between *M. f. fascicularis* and *M. f. aurea*. In the first, the hybrids occurred in the present time and so both Y-chromosome haplotypes still exist in the populations. The first hybrid event is potentially supported by the BMS population that has the morphology and Y-chromosome sequence of *M. f. aurea*, but the mtDNA sequence of the Indochinese *M. f. fascicularis*, supporting that *M. f. aurea* male recently migrated across mainland Thailand to the Thai Gulf islands. That the *M. f. fascicularis* Y-chromosome haplotype was not found in the BMS population may simply reflect the insufficient number of samples collected (two) to sample all common let alone rarer haplotypes in the population. The second scenario is that the hybrids occurred a long time ago but neither Y-chromosome haplotype attained any selected advantage over the other. Thus, the SRY, KRI, and WKC hybrids with the *M. f. aurea* Y-chromosome fragment did not attain any selective advantage over the *M. f. fascicularis* ones (Osada et al., 2010). Since the body mass and size of *M. f. aurea* were comparable to *M. f. fascicularis* (Bunlungsup et al., 2016), and so *M. f. fascicularis* males might be able to compete with introgressed *M. f. aurea* males to copulate with *M. f. fascicularis* females. Both Y-chromosome haplotypes can be retained in the population afterward. This is in contrast with the previously reported hybrids between *M. mulatta* and Indochinese *M. f. fascicularis* that possessed only the *M. mulatta* Y-chromosome haplotype (Bonhomme et al., 2009; Kanthaswamy et al., 2008; Stevison & Kohn, 2009; Tosi et al., 2002; Yan et al., 2011). *M. mulatta* males were approximately 35% heavier than those of *M. f. fascicularis* males (Hamada et al., 2005, 2008), and so *M. mulatta* males would likely on average possess a higher rank in the population with more chance to copulate or coerce *M. f. fascicularis*

females to copulate with them (Yan et al., 2011). Thus, the rhesus Y chromosome gene haplotype would be driven through the *M. mulatta* x *M. f. fascicularis* hybrid population. To test the two hybridization hypotheses between *M. f. fascicularis* x *M. f. aurea* would require the analysis of more genes, such as autosomal genes, microsatellite loci or SNP markers, and may also help elucidate when hybridization occurred. In addition, analysis of male specimens from the SRI population is required to understand the evolutionary scenario. From the phylogenetic trees based on the mtDNA and Y-chromosome gene sequences, some of the tool using macaques on the island in the Thai Gulf (KRI) was *M. f. aurea* x Indochinese *M. f. fascicularis* hybrids. Thus, genetics could play a role in the emerging tool use behavior in addition to environmental forces, morphological suitability and cognitive capability.

Finally, with respect to taxonomy, the mtDNA sequence analysis that demonstrated that *M. f. aurea* was separate from the *M. mulatta* and *M. f. fascicularis* cluster raises the question if the *M. f. aurea* group of macaques should be recognized as a distinct species (*M. aurea*) as opposed to a subspecies (*M. f. aurea*). They also showed distinctive morphological differences from those of nominotypical *M. fascicularis*, namely an infrazygomatic cheek hair pattern, darker face and pelage color, shorter tail, and no vertex crest hair (Fooden, 1995; Bunlungsup et al., 2016). Thus, when combining the morphological characters, genetic data, and limited geographical range with the typical tool use behavior (Gumert et al., 2009; Gumert & Malaivijitnond, 2013; Tan et al., 2015), *M. f. aurea* appears to be unique and distinctive from the other nine subspecies of *M. fascicularis* (Fooden, 1995). However, because they mostly live in mangrove forests, then habitat disturbance by humans and deforestation are a severe threat, as seen in Myanmar (San & Hamada, 2011) and the PNY Island, Thailand (Gumert et al., 2013). Thus, intensive research on this (sub) species should be conducted to establish a suitable conservation strategy before it becomes extinct, like in Bangladesh (Kabir & Ahsan, 2012).

CHAPTER VI

HYBRIDIZATION BETWEEN *Macaca fascicularis fascicularis* AND *M. f. aurea*

Introduction

Macaca fascicularis is one of the most well-known macaque species. They distribute in the wide habitat range throughout mainland and island in Southeast Asia including Bangladesh, Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia, Brunei, Indonesia, the Philippines and Nicobar Islands (India) (Fooden, 1995). According to the taxonomic status, *M. fascicularis* was divided into 10 subspecies based on the morphological and distribution area differences (Fooden, 1995). Among all of them, *M. fascicularis fascicularis* occupies the widest distribution range across southern part of Indochinese Peninsular and almost all of the core areas of insular region (Malaysia, Indonesia, the Philippines and Nicobar Islands) causing them become popularly using as a primate model in biomedical research. Another recognized subspecies is *M. fascicularis aurea*. Their distribution ranges from Rakhine state in Myanmar southward along Mergui Archipelago to Andaman Seacoast of southwestern Thailand (Fooden 1995; San & Hamada, 2009). Recently, this subspecies becomes a focal point of interest among other macaque species in the aspect of stone tool using behavior (Gumert et al., 2009, 2011, 2013; Gumert & Malaivijitnond, 2012, 2013; Malaivijitnond et al., 2007). Although their stone tool-use behavior was first discovered along Mergui archipelago since 1877 (Carpenter, 1887), there is no extensive research about this valuable behavior until a past decade when the report of tool using macaques in Thailand at Piak Nam Yai Island, Laem Son National Park was published in 2007 (Malaivijitnond et al., 2007).

With regard to the distribution areas between *M. f. fascicularis* and *M. f. aurea*, these two subspecies live closely around the Isthmus of Kra where it has been proposed as an intersubspecific contact zone and hybridization (Fooden 1995). Since *M. f.*

fascicularis is popularly used as a non-human primate model in biomedical research, their basic information including genetic characteristics, behaviors and evolutionary history (Fooden, 1995; Hamada et al., 2008, Girard-Buttoz et al., 2014; Bunlungsup et al., 2015; Liedigk et al., 2015), especially their hybridization with different macaque species *M. mulatta*, have been vastly studied (Bonhomme et al., 2009; Kanthaswamy et al., 2008; Osada et al., 2010; Bunlungsup et al., 2017). On the other hand, the research on *M. f. aurea* is mainly focused on their stone tool-use behavior. Therefore, the hybridization scenario between these two subspecies is under investigated so far. Up to now, only one hybridization study was reported (presented in Chapter V and Bunlungsup et al., 2015).

However, the genetic characteristics that was analyzed in the previous study is unilateral markers; mtDNA and Y-chromosome (SRY and TSPY) which traced only maternal and paternal inheritance, respectively. Based on the mtDNA phylogenetic tree (Chapter V), *M. f. fascicularis* was grouped with *M. mulatta* and separated from *M. f. aurea*. This indicated that *M. f. fascicularis* is evolutionarily closer to *M. mulatta* than the *M. f. aurea* which is currently classified as subspecies in the same *M. fascicularis* taxon. Therefore, the objective in this chapter is to explore further for the degree of genetic admixture of *M. f. aurea* in *M. f. fascicularis* using biparental molecular marker. Up to currently, there is no any extensive study on *M. f. aurea* genetics including the whole genome sequencing has been conducted. Therefore, the SNPs marker set which can be used to differentiate between *M. f. aurea* and *M. f. fascicularis* that are suitable for the hybridization has not been discovered yet. In this Chapter, instead of using autosomal SNPs (as in Chapter IV), autosomal STRs which were generally used to determine the genetic differentiation in many species of non-human primates (Bonhomme et al., 2009; Charpentier et al., 2012; Barelli., et al 2013; Kanthaswamy et al., 2013; Smith et al., 2014; Sukmak et al., 2014) were used as listed in Table 6.2.

Methods

Sample collection and DNA extraction

The species and subspecies of monkeys were identified mainly based on their cheek hair pattern, vertex of head crest, and pelage color (Fooden, 1995; Hamada et al., 2006, 2008) as mention in Chapter V. All DNA samples extracted from the blood and fecal samples were derived from the DNA bank of the Primate Research Unit, Chulalongkorn University (Table 6.1). Most of the fecal DNA samples were nearly used up in Chapter V and less than 50 µl of extracted DNA was remained for the Chapter VI's study.

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Faculty of Science in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University, Protocol Review No. 1423010.

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Table 6. 1 Region, locality, geographical coordinate, morphological subspecies identification and collected specimen in *M. f. fascicularis*, *M. f. aurea* and the hybrid between the two species. Bold letter indicates the hybrid. The subspecies was identified based on the morphological characters (Fooden, 1995; Hamada et al., 2006, 2008).

Region	Name of Location	GPS (North, East)	Taxon	Specimen
Thailand (Indochina)	1. Wat Khao Nor (KN)	15º 57′, 99º 52′	*M. f. fascicularis*	Blood
	2. Koram Island (KRI)	**12º 14′, 100º 00′**	**Mixed**	**Feces**
	3. Samroiyot National Park (SRY)	**12º 07′, 99º 57′**	**Mixed**	**Feces**
	4. Wat Khao Chong Krachok (WKC)	**11º 48′, 99º 48′**	**Mixed**	**Blood**
Thailand (Sunda)	5. Banmaisomboon School (BMS)	10º 51′, 99º 13′	*M. f. aurea*	Feces
	6. Wat Paknam Pracharangsarith (WPN)	9° 57′, 98° 35′	*M. f. aurea*	Blood
	7. Mangrove Forest Research Center (MFRC)	9º 52′, 98º 36′	*M. f. aurea*	Blood
	8. Piak Nam Yai Island (PNY)	9º 35′, 98º 28′	*M. f. aurea*	Feces
	9. Sirae Island (SRI)	**7º 54′, 161º 98′**	**Mixed**	**Feces**
	10. Khao Noi/Khao Tangkuan (KN/KTK)	7º 12′, 100º 35′	*M. f. fascicularis*	Blood
Myanmar	11. Bayin Nyi Temple (BNT)	16º 58′, 97º 29′	*M. f. aurea*	Blood
	12. Lampi Island (LPI)	10º 54′, 98º 12′	*M. f. aurea*	Feces
	13. Jarlan Island (Lord Loughbrough) (JLI)	10º 25′, 97º 56′	*M. f. aurea*	Feces
	14. Zadetkyi (ZDK)[a]	9º 58′, 98º 11′	*M. f. aurea*	Feces

STR genotyping

In total, 21 loci of STRs were tested in this study (Table 6.2). All markers are tetra□ nucleotide repeated motif which most of them were successfully amplified among several primate species such as gibbons (Barelli., et al 2013), macaques (Bonhomme et al., 2009; Kanthaswamy et al., 2013; Smith et al., 2014; Sukmak et al., 2014) and baboons (Charpentier et al., 2012). Each PCR reaction was carried out in 20 µl of the mixture containing 0.5U ExTaq DNA Polymerase (Takara Bio Inc., Shiga, Japan), 0.2 mM of each primer and 1 µl of DNA template in the manufacturer's buffer. One of the four fluorescent markers, 6-FAM, VIC, NED, and PET, were labeled at the 5'-end of the forward primer of each locus. STR amplification was performed with 45 cycles of 94°C for 25 sec, 50/55°C for 30 sec, and 72°C for 30 sec, and then followed by 72°C for 7min. PCR products were sent to the Macrogen, Korea for fragment analysis. Genotyping was performed using 3730xl (Applied Biosystems) and allele sizes were determined relative to an internal size stand of Liz 600 standard (Applied Biosystems).

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Table 6. 2 The 21 loci analyzed in this study. Bold letter indicates the loci that were unsuccessfully amplified from blood samples.

No.	**Locus name**	**Forward primer**	**Reverse primer**
1	D1S548	GAACTCATTGGCAAAAGGAA	GCCTCTTTGTTGCAGTGATT
2	D1S1656	GTGTTGCTCAAGGGTCAACT	GAGAAATAGAATCACTAGGGAACC
3	D2S1326	AGACAGTCAAGAATAACTGCCC	CTGTGGCTCAAAAGCTGAAT
4	D2S1329	TTGTGGAACCGTTCTCAAAT	GAAACTTCCACCTGGGTTCT
5	D3S1766	ACCACATGAGCCAATTCTGT	ACCCAATTATGGTGTTGTTACC
6	D3S1768	GGTTGCTGCCAAAGATTAGA	CACTGTGATTTGCTGTTGGA
7	D3S2459	CTGGTTTGGGTCTGTTATGG	AGGGACTTAGAAAGATAGCAGG
8	**D4S243**	**TCAGTCTCTCTTTCTCCTTGCA**	**TAGGAGCCTGTGGTCCTGTT**
9	D5S1457	TAGGTTCTGGGCATGTCTGT	TGCTTGGCACACTTCAGG
10	D6S501	GCTGGAAACTGATAAGGGCT	GCCACCCTGGCTAAGTTACT
11	D7S821	ACAAAACCCCAAGTACGTGA	TATGACAGGCATCTGGGAGT
12	**D7S1830**	**GTACATGATGGGCTGTCCTC**	**GATACATACTGCCAATAAATCACA**
13	D8S1106	TTGTTTACCCCTGCATCACT	TTCTCAGAATTGCTCATAGTGC
14	D10S611	CATACAGGAAACTGTGTAGTGC	CTGTATTTATGTGTGTGGATGG
15	D10S1432	CAGTGGACACTAAACACAATCC	TAGATTATCTAAATGGTGGATTTCC
16	D11S2002	CATGGCCCTTCTTTTCATAG	AATGAGGTCTTACTTTGTTGCC
17	D13S321	TACCAACATGTTCATTGTAGATAGA	CATACACCTGTGGACCCATC
18	D14S306	AAAGCTACATCCAAATTAGGTAGG	TGACAAAGAAACTAAAATGTCCC
19	**D18S851**	**CTGTCCTCTAGGCTCATTTAGC**	**TTATGAAGCAGTGATGCCAA**
20	D20S206	TCCATTATTCCCCTCAAACA	GGTTTGCCATTCAGTTGAGA
21	AGAT006	AGTGGATCGATAGATTGACAGATG	TCAGGTGACAGCCAAGTCAATTCA

Results

STRs analysis

Eighteen out of 21 loci were successfully amplified from DNA obtained from blood samples as shown in Figure 6.1(a). However, low success rate in PCR amplification and low intensity of bands were obtained from fecal DNA samples (Figure 6.1(b) and 6.1(c)), partly due to poor quality and small amount of DNA. As mentioned in the method that the remaining amount of fecal DNA sample was small. Therefore, the PCR amplification could not be repeated.

Among seven selected fecal samples which were PCR amplified on trial (Figure 6.1(a) and 6.1(b)), none of them provided a complete set of the 18 loci PCR products. Although one fecal sample (no.4) showed high successful amplification rate and 11 of 21 loci were obtained, only one potential locus showed a high efficiency in discriminating between *M. f. fascicularis* and *M. f. aurea* which was not enough for further step of the analysis.

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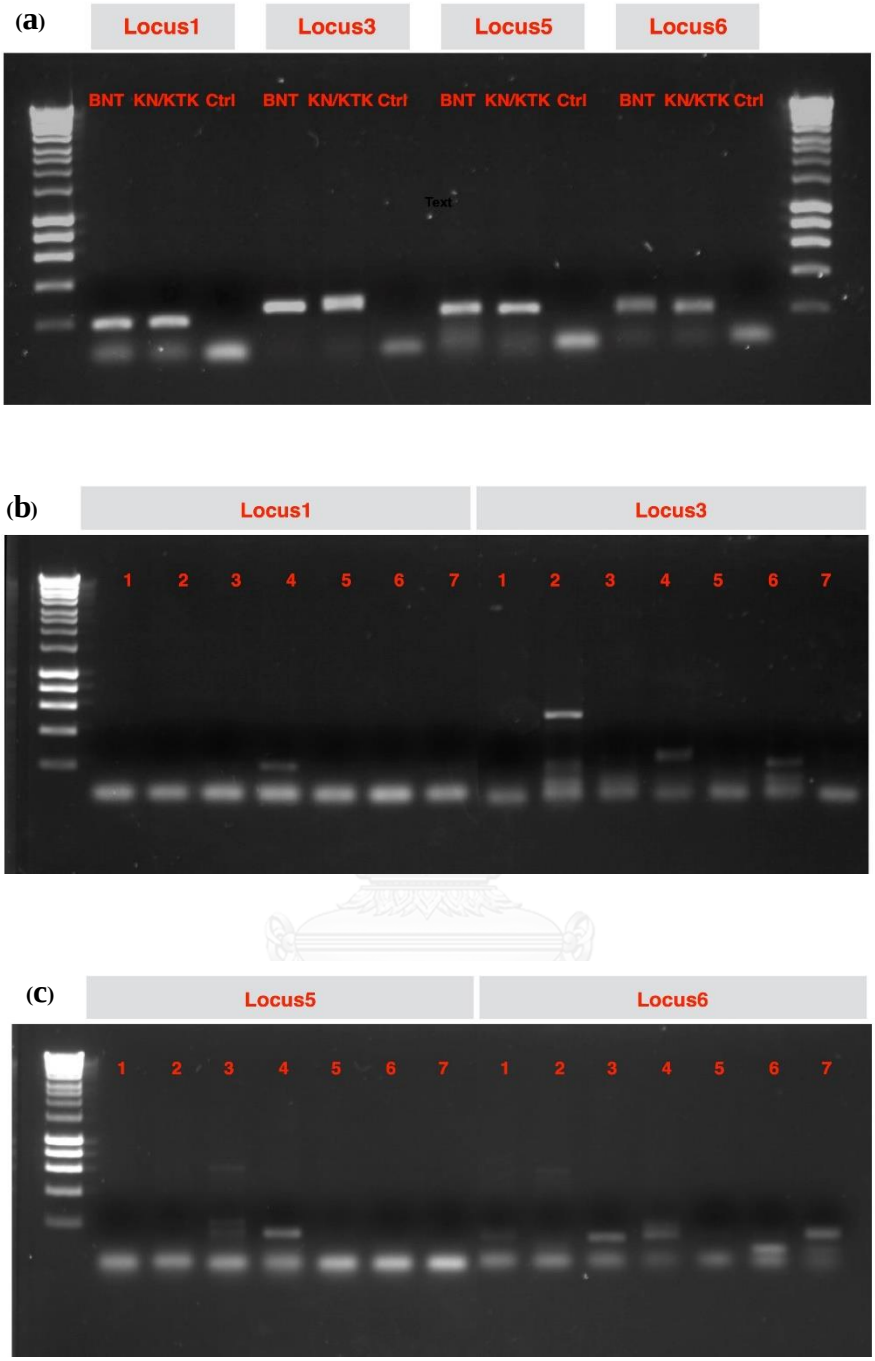

Figure 6. 1 Compared between amplification results of loci 1, 3, 5 and 6 from (a) blood samples and (b and c) fecal samples on 1.5% agarose gel electrophoresis. BNT, KN/KTK and Ctrl refer to Bayin Nyi Temple (representative of *M. f. aurea*), Khao Noi/Khao Tangkuan (representative of *M. f. fasicularis*) and negative control, respectively. Number 1-3, 4-5 and 6-7 refer to fecal samples from Samroiyot National Park, Koram Island and Piak Nam Yai Island which are hybrid individuals, respectively

Discussion and recommendations

The degree of genetic admixture of *M. f. aurea* in *M. f. fascicularis* was determined using STR (21 autosomal loci) markers. Although 18 out of 21 loci were successfully amplified from the blood samples across these two *M. fascicularis* subspecies, none of the fecal samples could provide a complete set of the 18 loci PCR products. Besides, only fecal samples were collected from three of the four morphological hybrid populations (KRI, WKC, SRI), and thus the quality of PCR product was poor and could not proceed to the next step of the analysis. Gagneus, Boesch, & Woodruff (1997) used hair sample for STRs amplification and revealed that allelic dropout (the occurrence when one of the allele was not amplified in PCR) is one of the severe problems which caused genotyping error. About 31% of false homozygous were found in single hair amplification, but it can be reduced to be lower than 0.05 in three separate amplifications. Therefore, several sets of PCR amplification are required for each analyzed sample to gain the reliability of genotyping data. Unfortunately, remaining amount of fecal DNA was not enough to proceed to those steps.

For the population genetic analysis, representative alleles in any particular population are depended on allele characteristics (very rare, rare, and common) and sampling size. Hale, Burg & Steeves (2012) suggested that the increase in sample size could decrease the variability of allele frequency and expected heterozygosity (HE) among replicates. Those variabilities were minimal if the sample size was higher than 25-30. As such, at least 25-30 macaque individuals per population are required for the analysis of microsatellite allele frequencies. As only few fecal samples (individuals) of hybrid populations were collected in this study, it is not beneficial to test the gradient of the genetic admixture between these two closely related subspecies using these STR markers.

With regard to the mtDNA phylogenetic analysis (result in Chapter V), *M. f. aurea* was separated from *M. f. fascicularis* and *M. mulatta* clade, thus *M. f. aurea* should

possess some genetic distinctive and may evolve independently from their conspecific *M. f. fascicularis* (Matsudaira et al., personal communication). One on-going project of our research team is sequencing and analyzing the whole genome of *M. f. aurea*. Hopefully, in the near future, more suitable markers should be discovered, and thus more intensive research on *M. f. aurea* evolution including the hybridization scenario with *M. f. fascicularis* should be feasible. Exploring their genetic characteristics using high-throughput method could help us to verify whether the genetic characteristics of *M. f. aurea* is indeed being different from *M. f. fascicularis* and other subspecies of *M. fascicularis*. In addition, screening the whole genome sequence of *M. f. aurea* may discover some related cognitive genes which play a vital role together with environment on stone manipulation in this macaque subspecies.

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CHAPTER VII
GENERAL DISCUSSION AND CONCLUSIONS

M. f. fascicularis is one of the most popularly used non-human primate models in biomedical research and thus their genetic characteristics are a major concern during the past decades (Stevison & Kohn, 2008; Satkoski et al., 2008a; Kanthaswamy et al., 2006; Kawamoto et al., 2008). *M. f. fascicularis* lived sympatrically with *M. mulatta* at 15-20° N in natural habitats and hybrids of these two species have been reported causing additional more complications to the genetic status of *M. f. fascicularis*. Their hybrid zone has been proposed to cover four countries in Indochina including Myanmar, Thailand, Laos and Vietnam (Fooden, 2000; Hamada et al., 2006). Apart from hybridization between these two species, *M. f. fascicularis* and *M. f. aurea* live in adjacent natural habitats and have been reported to hybridize near the Isthmus of Kra, in southern Thailand (Fooden, 1995). In contrast to the many studies of hybrids between *M. f. fascicularis* and *M. mulatta*, there is only one report by Fooden (1995) on the morphological characters of intersubspecific hybrids of *M. f. fascicularis* and *M. f. aurea*. Because hybrid offspring of these macaques either between *M. f. fascicularis* and *M. mulatta* or between *M. f. fascicularis* and *M. f. aurea* are fertile and can pass their mixed genetic ancestry to their descendants, this further complicates an understanding of their genomes.

Natural introgressive hybridization has been shown to generate genetic enrichment as well as the formations of a new taxon and thus, considered as a vital mechanism in primate evolution (Arnold & Meyer, 2006). Therefore, the study of interspecific (*M. f. fascicularis* × *M. mulatta*) and intersubspecific (*M. f. fascicularis* × *M. f. aurea*) hybridization should provide an extensive knowledge both in understanding the genetic characteristics and composition as well as the evolutionary scenario of *M. fascicularis*. Although there were extensive studies over the past decade on the hybridization between *M. fascicularis* and *M. mulatta* based on physiological

(Malaivijitnond et al., 2008), morphological (Hamada et al., 2008; Hamada et al., 2006; Jadejaroen et al., 2015) and genetic (Kanthaswamy et al., 2008; Osada et al., 2010; Satkoski et al., 2013; Tosi et al., 2002) characteristics of both these parental and hybrid populations, the perspective of these species' hybridization scenario still remains incomplete. One of the reasons may be due to the lack of specimens from animals of known origins living in Thailand which represents a geographical zone of hybridization between the two sister species of macaques as well as the two subspecies of *M. fascicularis*. Therefore, the hybridization at both the interspecific and intersubsepcific levels were assessed and analyzed in this study based on collected specimens of *M. mulatta*, *M. f. fascicularis* and *M. f. aurea* throughout Thailand and vicinity. In this study, three distinct classes of genetic markers were selected for the analyses which was aimed at exploring the different scenarios of hybridization processes. Since macaque females live typically in their natal group (female philopatry) while the males migrate to other groups after reaching sexual maturity (male dispersal), the use of mtDNA, and Y-chromosome linked markers were selected to answer questions about the hybridization and male introgression of the groups. Autosomal SNPs and STRs were selected to answer the question about the degree of nuclear genetic admixture between the two species (*M. f. fascicularis* × *M. mulatta*) and two subspecies (*M. f. fascicularis* × *M. f. aurea*), respectively.

Based on previous studies (Tosi et al., 2002; Tosi & Coke, 2007) which assessed the mtDNA and Y-chromosome phylogenies of *M. mulatta* and *M. fascicularis*, a one directional model of gene flow mediated by male *M. mulatta* into *M. fascicularis* populations was proposed. That study also concluded that the hybridization terminated at the Klong Marui fault. Using mtDNA (HVSI) and Y-chromosome (*TSPY* and *SRY*) genes in this study, the uni-directional genetic introgression was confirmed. However, more precise termination area of genetic introgression was revealed here. *M. mulatta* gene flow went southward further than the Isthmus of Kra and was terminated between SSD (Chumphon) and WSK (Phang Nga). Since SSD and WSK are located on the

southern side of the Isthmus of Kra, that the Isthmus of Kra does not represent a sharp restrictive barrier between *M. mulatta* and *M. fascicularis*, but rather it causes gradual changes in species distribution similar to other taxa (Hughes et al., 2011). Apart from the two major clades of mtDNA phylogenies; continental and insular *M. f. fascicularis*, Harihara et al., (1988) and Liedigk et al. (2015), based on the analyses of intensive specimen collections in Thailand, demonstrated that four subclades have clearly diverged within the continental *M. f. fascicularis* clade (Sundaic Thai Gulf, Vietnam, Sundaic Andaman Sea Coast and Indochina). These four subclades correspond with significant zoogeographical barriers such as the Sunda land-bridge, the Isthmus of Kra, and the Nakhon Si Thammarat and Phuket mountain ranges. Among these four subclades, the Sundaic Thai Gulf subclade has never been reported elsewhere to the best of our knowledge. Considering that Thai Gulf and Andaman Sea Coast are both located on the Sundaic region of Thailand, and the discovery of the separation of Thai Gulf subclade from other three clades of Indochina, Vietnam and Sundaic Andaman Sea Coast, this new information resembles a piece of a missing jigsaw that could potentially resolve the link between Malaysian/ Sumatran and Indochinese *M. fascicularis* which is being examined for several years by other researchers (Blancher et al., 2008; Abdul-Latiff et al., 2014a).

Consistent with mtDNA and Y-chromosome analyses, SNP genotype data confirmed the hybridization and genetic admixture between *M. mulatta* and *M. f. fascicularis*. Contrarily to the uni-directional gene flow acquired from Y-chromosome analysis, admixture bar blot supported a biased bi-directional hybridization (Hamada et al., 2016; Kanthaswamy et al., 2010; Osada et al., 2010), in which the genetic introgression from *M. mulatta* to *M. f. fascicularis* was greater than that from *M. f. fascicularis* into *M. mulatta*. The maximum level of the genetic admixture between the two species was found in the proposed hybrid zone (15-20 °N) and these levels declined gradually and proportionately with the geographic distance. The genetic admixture between *M. mulatta* into *M. f. fascicularis* was restricted somewhere between KNKTK,

southern Thailand and Singapore/Sarawak, ranging for at least 10 degrees of latitude, while the genetic introgression from *M. f. fascicularis* to *M. mulatta* was restricted at somewhere between BSS, northeast Thailand and SMT, Myanmar which was only about 4 degrees of latitude. This asymmetrical hybridization observed here may be driven by the selective advantage of *M. mulatta* over *M. fascicularis*, especially considering their morphological characteristics where *M. mulatta* are larger and heavier in size than *M. fascicularis* (Hamada et al., 2006). Given their larger size it is possible that either male *M. mulatta* coerce female *M. fascicularis* to mate with them or female *M. fascicularis* prefer to mate with the larger male *M. mulatta*. Moreover, *M. mulatta* are seasonal breeders and thus give reproduce seasonally (Weinbauer et al., 2008) while *M. fascicularis* reproduce throughout the year with a reproductive peak period that depends on food availability (Kavanagh & Laursen, 1984; van Schaik & Noordwijk, 1985). Consequently, female *M. mulatta* is generally less frequently receptive to mating with both the conspecific or non-conspecific males compared to female *M. fascicularis*.

The hybridization study between *M. f. fascicularis* and *M. f. aurea*, mtDNA and Y-chromosome phylogeny supports the hybridization hypothesis (Fooden, 1995) that male *M. f. aurea* introgressed into *M. f. fascicularis* populations. Based on mtDNA phylogeny from this study, all hybrids discovered in this study can be divided into 2 groups; Indochinese (SRY, KRI, WKC, and BMS) and Sundaic (SRI) hybrids. Since the oldest haplotype of *M. f. aurea* is from mainland Myanmar, two hybridization routes have been proposed in this study. First route, *M. f. aurea* from mainland Myanmar migrated southward along Mergui archipelago to Andaman Sea Coast and *M. f. aurea* males hybridized with Sundaic *M. f. fascicularis* females (represented by SRI population). Second route, Along the migration to Mergui Archipelago, *M. f. aurea* migrated northeastward across the low altitude area of the Dawna range (San & Hamada, 2011) to mainland Thailand and the islands on the Thai Gulf and *M. f. aurea* males hybridized with Indochinese *M. f. fascicularis* females (represented by the SRY, KRI, WKC, and

BMS populations).

Combining the estimated time based on mtDNA topology in this study together with previous studies, the evolutionary scenario of *M. f. fascicularis*, *M. f. aurea* and *M. mulatta* was drawn. Around 2.5-0.95 MYA (Figure 7.1), proto-*M. f. aurea and* proto-*M. f. fascicularis* diverged from each other, and then proto-*M. f. aurea* hybridized with a population of *sinica* species group at some area in Myanmar or Bangladesh (at the present time; Matsudaira et al., pers. com.). After that, at some point, *M. f. fascicularis* expanded throughout mainland and island in Southeast Asia. About 1.19 MYA (Figure 7.1), gene flow between insular monkeys and other regions was restricted and the insular *M. f. fascicularis* diverged from other *M. f. fascicularis* populations. This might be a result of the rise of sea level which occurred many times during the last 2 MYA (Raymo et al., 2011). Around 1.07 MYA (Figure 7.1), the continental *M. f. fascicularis* were separated into the Indochinese populations (in the north), which later became the founders of the Indochinese, Vietnamese and Sundaic Andaman populations, and the Sundaic populations (in the south) which became the founders of Sundaic Thai Gulf populations. This diversion may be attributed to the Isthmus of Kra, the largest biogeographical barrier in the region, which has also influenced the distributions of many other animal and plant species (Denduangboripant & Cronk, 2000; Inger & Voris, 2001; Hughes et al., 2003, 2011). After that, around 0.95 MYA (Figure 7.2), *M. f. aurea* diverged into, at least, two regions of mainland Myanmar and Mergui archipelago (Matsudaira et al., pers. comm.). At 0.62-0.73 MYA, Indochinese *M. f. fascicularis* founders diverged rapidly into three sub-groups of Indochinese, Vietnam and Andaman seacoast (Figure 7.2). This time point corresponds to the earlier interglacial period when the sea level had started to increase at *ca.* 0.63 MYA (Stirling et al., 2001). Presumably, the close genetic relationship among these three sub-groups might occur during the glacial period when the sea level began to drop (up to 60 meters lower than the present-sea level; Lambeck, Esat, & Potter, 2002; Stirling et al., 2001; woodruff, 2010) and the

land bridge was emerged and connected between Indochina (southern Vietnam/Cambodia) and Sunda (southernmost Thailand/northern peninsular Malaysia) regions (Sathiamurthy & Voris, 2006 and also Figure 2.6-2.9 in Chapter II). The emerged land bridge together with a cooler climate in the northern parts forced the Indochinese *M. f. fascicularis* to emigrate in two different directions to the Sundaic region. One direction was directly southwardly to the Andaman Sea Coast and another was eastwardly through Vietnam and southwardly to the peninsular Malaysia and northern Sumatra (Figure 7.3). At that time, the Sundaic *M. f. fascicularis* should be restricted to the Thai gulf (i.e. forming the Thai Gulf subclade in the mtDNA phylogenetic tree) by the Nakhon Si Thammarat mountain and Phuket ranges which allowed Indochinese *M. f. fascicularis* to disperse only toward the Andaman Sea Coast. This migration scenario could clarify unanswered questions from the previous mtDNA studies why the Indochinese *M. f. fascicularis* belonged to the same clade of the animals inhabiting peninsular Malaysia (Abdul-Latiff et al., 2014a) and Sumatra (Blancher et al., 2008), respectively.

However, the exact time of the hybridization between *M. mulatta* and *M. f. fascicularis* has yet been obscured. Based on SNPs analysis, Osada et al. (2010) suggested that the hybridization occurred over 1 MYA or during the middle to late Pleistocene, while the result of STRs analysis indicated more recent time of about 3,400 year ago (Bonhomme et al., 2009). Since only the Indochinese *M. f. fascicularis* gained Y-chromosome haplotype of *M. mulatta*, while those of Andaman populations possessed their own Y-chromosome haplotype (Sundaic *M. f. fascicularis*), the hybridization event should have started after the divergence of Indochinese founders (after 0.62-0.73 MYA) into three sub-groups of Indochinese, Vietnam and Andaman seacoast (Figure 7.2). Although the exact time of the interspecific hybridization cannot be estimated in this study, using mtDNA (HVSI) and Y-chromosome (*TSPY* and *SRY*) markers indicated a uni-directional gene flow of male *M. mulatta* into *M. fascicularis* populations which was terminated between SSD (Chumphon) and WSK (Phang Nga)

(Tosi et al., 2002; Tosi & Coke, 2007). On contrarily, a biased bi-direction gene flow was found after using SNP analysis and the gradient of genetic admixture was detected. Their genetic admixture was maximized at the proposed hybrid zones (15-20° N), and declined gradually in relation to the distance from the hybrid zone and the zoogeographical barriers.

For the intersubspecific hybridization between *M. f. fascicularis* and *M. f. aurea*, since both types of *M. f. fascicularis* and *M. f. aurea* Y-chromosome were observed in all Indochinese hybrid (SRY, WKC and KRI) populations, the two intersubspecific hybridization scenarios are proposed (Figure 7.2). First, the hybridization occurred long time ago, but after the divergence of Indochinese *M. f. fascicularis* founders (after 0.62-0.73 MYA); however, there is no selective pressure and both types of Y-chromosome could co-exist. Second, the intersubspecific hybridization occurred recently and neither *M. f. fascicularis* nor *M. f. aurea* Y-chromosome has been eliminated yet. Moreover, the two hybridization routes were also proposed; (i) after male *M. f. aurea* migrated southwardly along Mergui archipelago, some of them introgressed into Sundaic *M. f. fascicularis* populations and produced Sundaic (SRI) hybrids and (ii) during the migration along Mergui Archipelago, some of male *M. f. aurea* migrated eastwardly across the low altitude of Tanintharyi range,introgressed into Indochinese *M. f. fascicularis* populations and produced Indochinese (SRY, WKC and KRI) hybrids (Figure 7.4).

In addition to a new perspective on *M. f. fascicularis* x *M. f. aurea* hybridization events, this thesis has discovered that *M. f. aurea* are very genetically distinctive from their conspecific *M. f. fascicularis*. Since *M. f. aurea* do not belong to the *M. f. fascicularis*-*M. mulatta* clade after the mtDNA analysis, this leads to a question about the genetic composition of *M. f. aurea* and their taxonomic status. Unfortunately, STRs genotyping was not successfully done in this study, the evolutionary history of *M. f. aurea* awaits to be resolved in the future.

In conclusion, this study divulges how complication of genetic characteristics and evolutionary history of *M. f. fascicularis* is. Thus, it underscores the importance of information on taxon's species or subspecies and geographical origins of *M. f. fascicularis* when they will be used as a non-human primate model for biomedical research.

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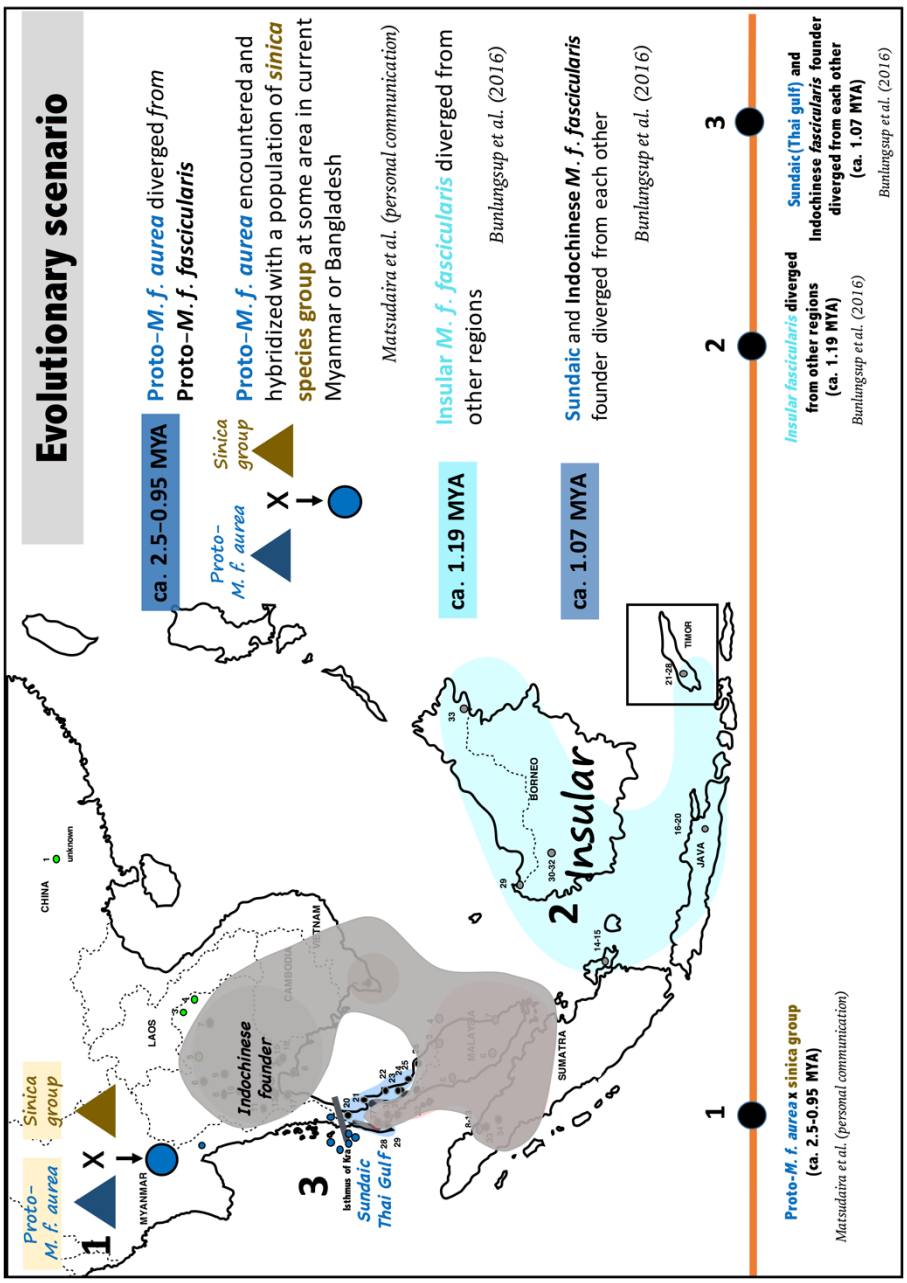

Figure 7.1 Evolutionary scenario of *M. f. fascicularis*, *M. f. aurea* and *M. mulatta*. Number 1-3 indicate the sequence of each event.

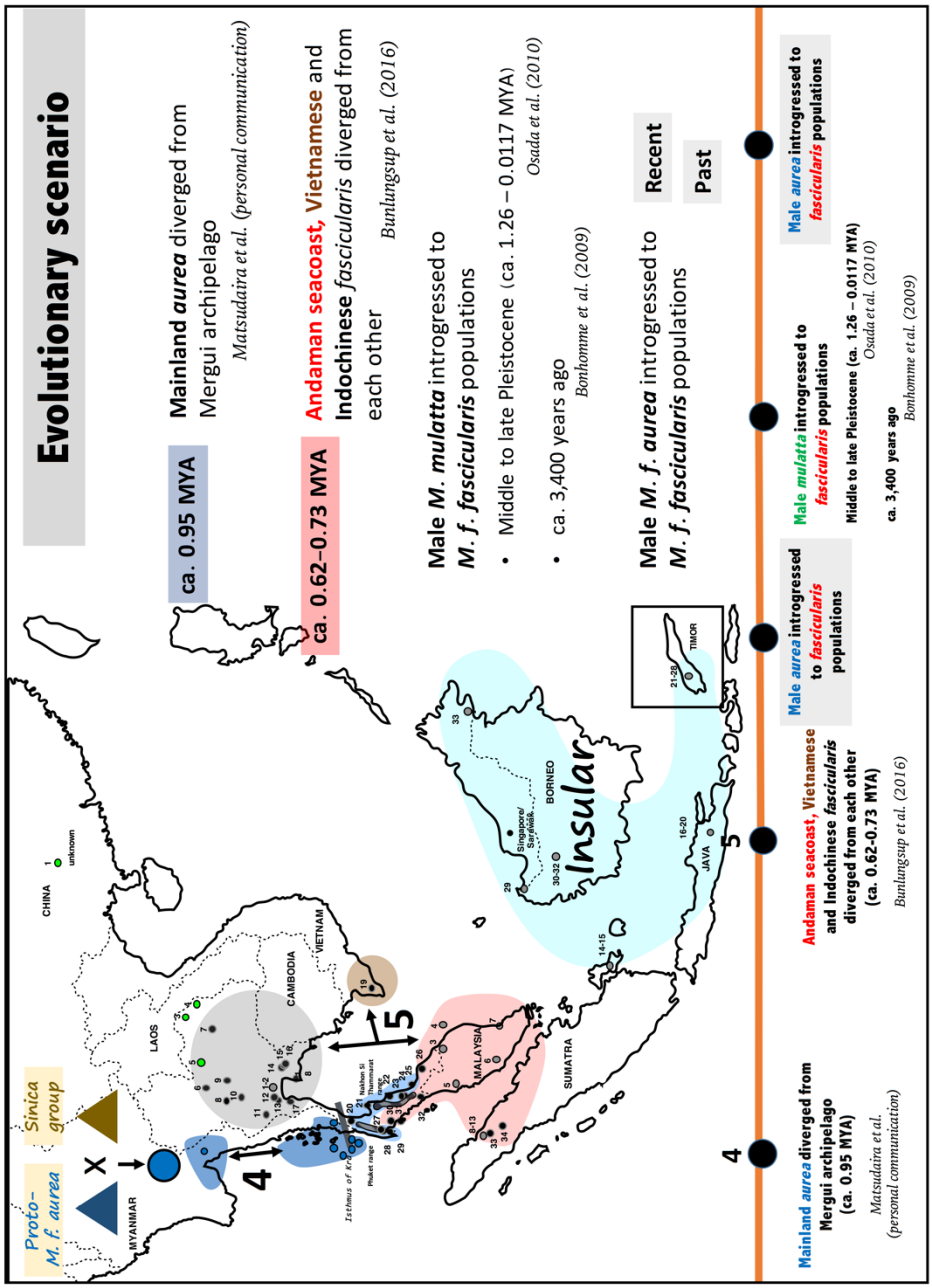

Figure 7.2 Evolutionary scenario of *M. f. fascicularis*, *M. f. aurea* and *M. mulatta*. Number 4 and 5 indicate the sequence of each event.

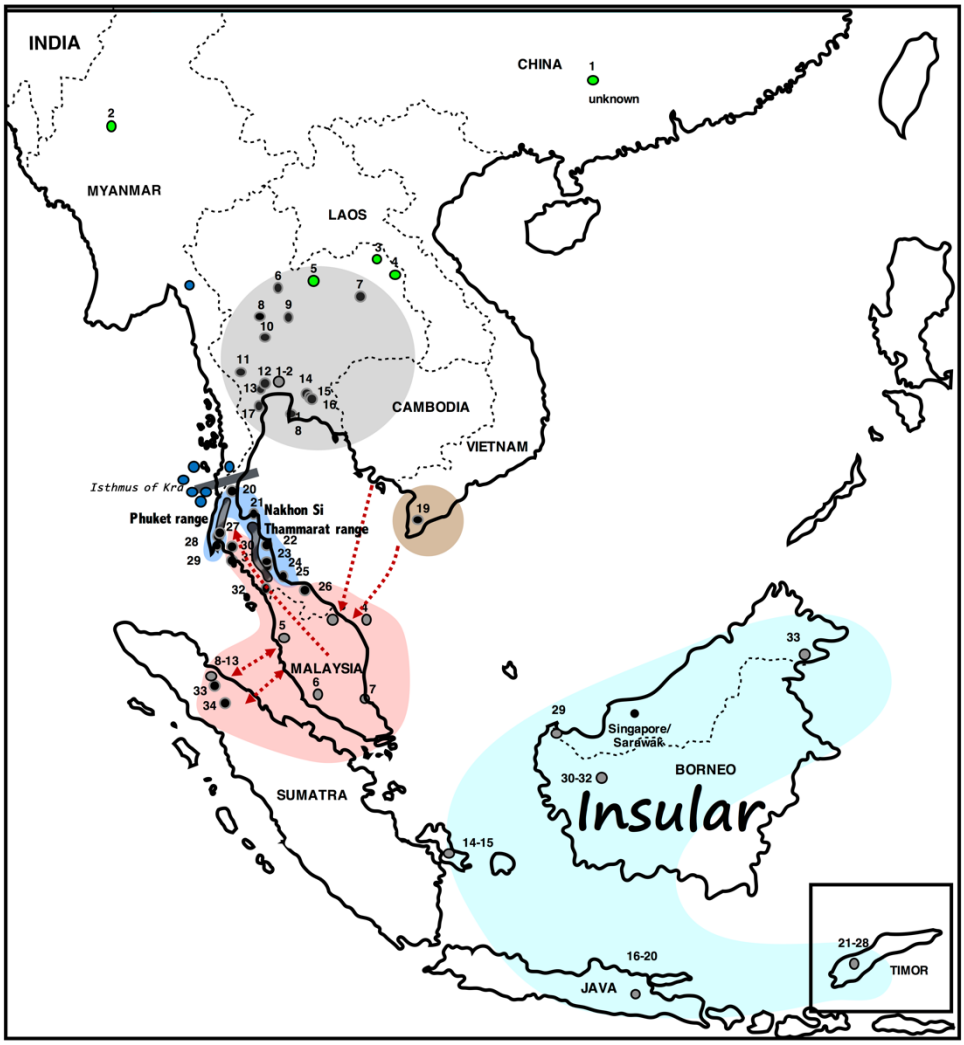

Figure 7.3 The migratory scenario of *M. f. fascicularis* from mainland Indochina, across land bridge, to the peninsular Malaysia and Sumatra during glacial period which leads to a close phylogenetic relationship between Indochina (black paint), Vietnamese (brown paint) and Andaman seacoast (red paint).

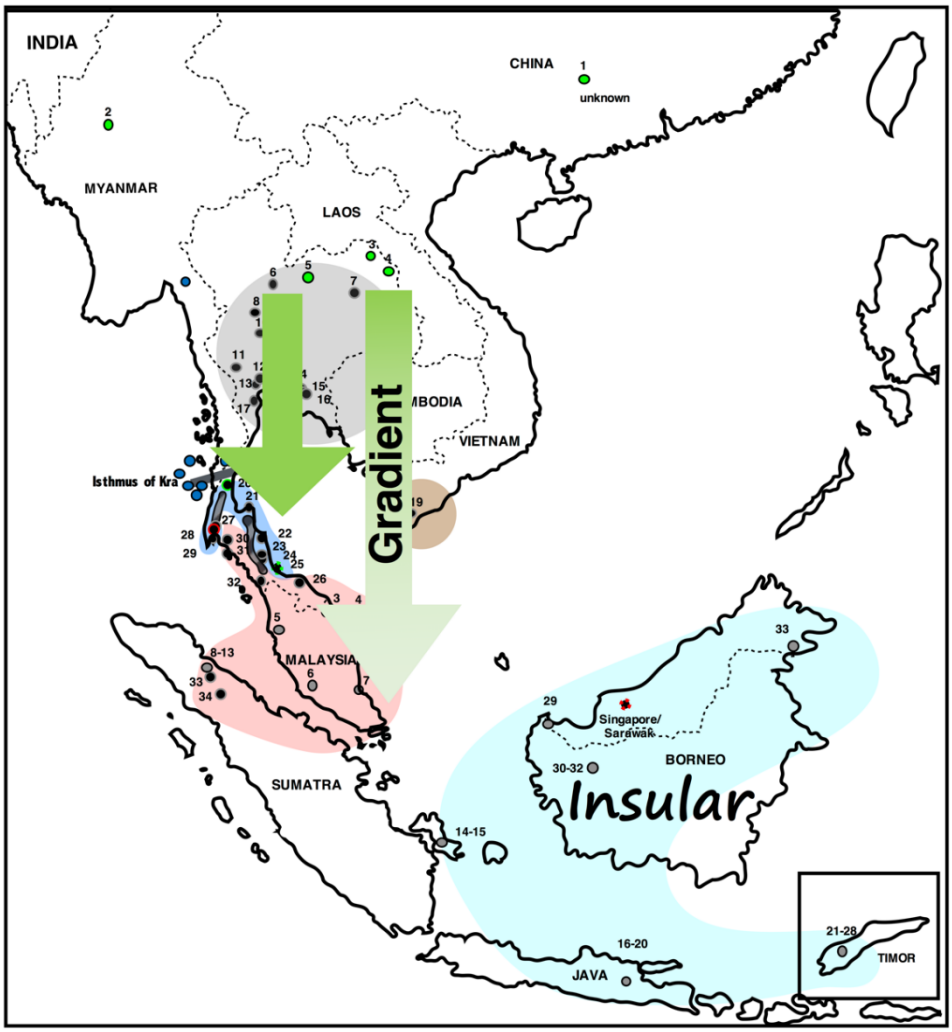

Figure 7. 4 The hybridization scenario between *M. f. fascicularis* and *M. mulatta*. Green color indicates genetic introgression from *M. mulatta* to *M. f. fascicularis* Using mtDNA and Y-chromosome, it indicates that male *M. mulatta* gene flow was terminated between Chumporn, no.20 (block circle with green border) and Songkla, no. 28 (black circle with red border) provinces. Using SNPs analysis, it indicates that *M. mulatta* gene flow was restricted between Songkla, no. 25 (black circle with green dot border) and Singapore/Sarawak (black circle with red dot border).

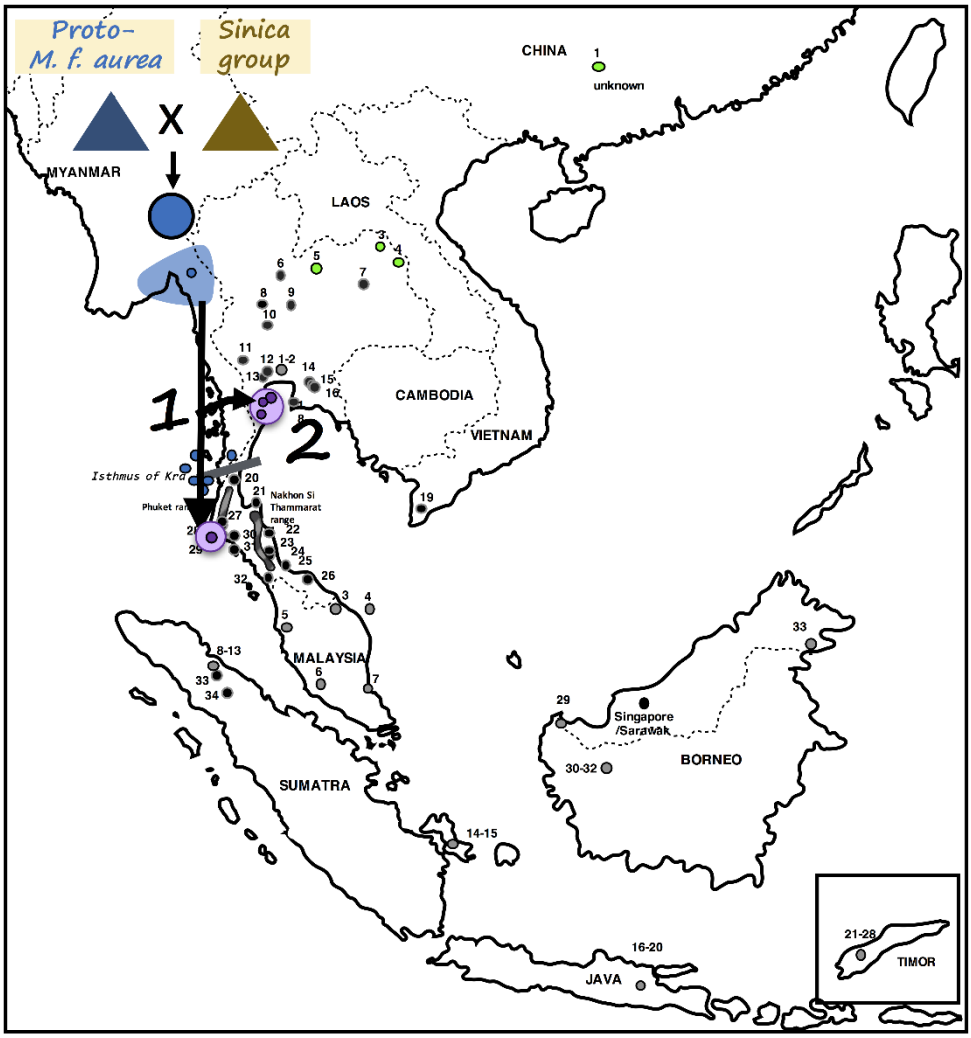

Figure 7. 5 The hybridization scenario between *M. f. fascicularis* and *M. f. aurea*. Black arrow indicates two hybridization routes; (1) *M. f. aurea* male moved southward to Andaman sea coast which led to the Sundaic hybrids, and (2) *M. f. aurea* moved eastward across Tanintharyi mountain range which led to Indochinese hybrids.

Recommendations

1. The whole genome sequencing of *M. f. aurea* should be performed. Thus, the large-scale of genetic information should be gained and the more suitable genetic markers, especially autosomal SNPs set, which are useful to determine the level of genetic admixture between *M. f. fascicularis* and *M. f. aurea*, should be acquired.

2. To draw the fine scenario of the termination of gene flow from *M. mulatta* to *M. f. fascicularis*, specimens from other locations, for example, peninsular Malaysia, should be collected.

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VITA

Miss Srichan Bunlungsup was born on December 8th, 1990 in Bangkok, Thailand. She received her Bachelor degree of Science (second class honor) from Faculty of Veterinary Technology, Kasetsart University, Bangkok in 2011. Later on, she continued her Ph.D. study in Zoology Program at the Department of Biology, Faculty of Science, Chulalongkorn University. She was fully supported by Thailand Research Fund through the Royal Golden Jubilee Ph.D. (RGJ Ph.D.) program during her Ph.D. study. She also received the research funding from the 90th Anniversary of Chulalongkorn University Fund.

In 2014, she was awarded a short-term scholarship from Japan Student Services Organization (JASSO), The Ministry of Education, Culture, Sports, Science and Technology, Japan to conduct her research under the supervision of Professor Dr. Gen Watanabe and Associate Professor Dr. Kentaro Nagaoka at the Veterinary Physiology Laboratory, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Japan. Subsequently, she was also awarded a Cooperative Research Program Scholarship from Primate Research Institute of Kyoto University for two times, in 2014 and 2015, to conduct her experiments there under supervision of Associate Professor Dr. Hiroo Imai. In 2016, she was supported by the RGJ-Ph.D. program to conduct her 6-month Ph.D. research under supervision of Associate Professor Dr. Sree Kanthaswamy at Evolutionary & Forensic Genetics Laboratory, School of Mathematical and Natural Sciences, Arizona State University (ASU), USA and Professor Dr. David Glenn Smith from the Department of Anthropology Evolutionary Wing, University of California Davis.

She performed the first international oral presentation at the 4th Asian Primate Symposium which was held on August 20th – 22nd, 2014 at Bogor University, Indonesia. Her second international oral presentation was at the 31st Congress of the Primate Society of Japan which was held on July 18th – 20th, 2015 at Kyoto University, Japan. During her stay at ASU in 2016, she had a chance to give a special talk for undergraduate students in Forensic Biology class. Besides the oral presentation, she did poster presentation once in the Symposium on Primate Diversity in East and Southeast Asia which was held on December 15th, 2015 at Chulalongkorn University.

Srichan Bunlungsup could publish two papers during her Ph.D. study as follows;

1. Bunlungsup, S., Imai, H., Hamada, Y., Gumert, M.D., San, A.M., & Malaivijitnond, S. (2015). Morphological characteristics and genetic diversity of Burmese long-tailed macaques (Macaca fascicularis aurea). American Journal of Primatology, 78, 441-455. (Q1 Zoology, impact factor 2015 = 2.103)

2. Bunlungsup, S., Imai, H., Hamada, Y., Matsudaira, K., & Malaivijitnond, S. (2017). Mitochondrial DNA and two Y-chromosome genes of common long-tailed macaques (Macaca fascicularis fascicularis) throughout Thailand and vicinity. American Journal of Primatology, 79, 1-13. (Q1 Zoology, impact factor 2015 = 2.103)