DEGREE OF GENETIC ADMIXTURE IN HYBRID POPULATIONS BETWEEN *Macaca fascicularis fascicularis* AND *M. f. aurea* IN ASSOCIATION WITH STONE TOOL-USE BEHAVIOR

Mr. Poompat Phadphon

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ระดับของการผสมทางพันธุกรรมในประชากรลูกผสมระหว่าง *Macaca fascicularis fascicularis* และ *M. f. aurea* ที่สัมพันธ์กับพฤติกรรมการใช้เครื่องมือหิน

นายภูมิพัฒน์ ผาดโผน

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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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Thesis Title	DEGREE OF GENETIC ADMIXTURE IN HYBRID POPULATIONS BETWEEN *Macaca fascicularis fascicularis* AND *M. f. aurea* IN ASSOCIATION WITH STONE TOOL-USE BEHAVIOR
By	Mr. Poompat Phadphon
Field of Study	Zoology
Thesis Advisor	Professor SUCHINDA MALAIVIJITNOND, Ph.D.
Thesis Co Advisor	Professor Sreetharan Kanthaswamy, Ph.D.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

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(Professor SUCHINDA MALAIVIJITNOND, Ph.D.)

.....
Thesis Co-Advisor
(Professor Sreetharan Kanthaswamy, Ph.D.)

.....
Examiner
(Assistant Professor NATAPOT WARRIT, Ph.D.)

.....
Examiner
(Assistant Professor SITTIPORN PATTARADILOKRAT, Ph.D.)

.....
Examiner
(Professor SOMSAK PANHA, Ph.D.)

.....
External Examiner
(Professor Yuzuru Hamada, Ph.D.)

ภูมิพัฒน์ ผาดโผน : ระดับของการผสมทางพันธุกรรมในประชากรลูกผสมระหว่าง *Macaca fascicularis fascicularis* และ *M. f. aurea* ที่สัมพันธ์กับพฤติกรรมการใช้เครื่องมือหิน. (DEGREE OF GENETIC ADMIXTURE IN HYBRID POPULATIONS BETWEEN *Macaca fascicularis fascicularis* AND *M. f. aurea* IN ASSOCIATION WITH STONE TOOL-USE BEHAVIOR) อ.ที่ปรึกษาหลัก : ศ. ดร.สุจินดา มาลัยวิจิตรนนท์, อ.ที่ปรึกษาร่วม : ศ. ดร.Sreetharan Kanthaswamy

ลิงหางยาวชนิดย่อยพม่า (Burmese long-tailed macaque; *Macaca fascicularis aurea*) เป็นลิงโลกเก่าเพียงชนิดเดียวที่แสดงพฤติกรรมการใช้เครื่องมือหินเพื่อเข้าถึงอาหารที่มีเปลือกแข็งหุ้ม ลิงหางยาวชนิดย่อยพม่ามีการแพร่กระจายใกล้ชิดกับลิงหางยาวชนิดย่อยธรรมดา (common long-tailed macaque; *M. f. fascicularis*) ในบริเวณตอนใต้ของประเทศไทยที่ละติจูด 8-12 องศาเหนือ และมีรายงานการผสมข้ามสายพันธุ์ระหว่างลิงสองชนิดย่อยในบริเวณดังกล่าว และพบพฤติกรรมการใช้เครื่องมือหินในลิงลูกผสมเช่นเดียวกัน แต่กลับไม่พบพฤติกรรมดังกล่าวในลิงหางยาวชนิดย่อยธรรมดา ทั้งในสภาพธรรมชาติหรือในกรงเลี้ยง จึงเป็นที่น่าสนใจว่าปัจจัยทางพันธุกรรมมีบทบาทต่อพฤติกรรมการใช้เครื่องมือหินหรือไม่ ดังนั้นการศึกษาในครั้งนี้จึงทำการสำรวจประชากรลิงหางยาวชนิดย่อยพม่า ลิงหางยาวชนิดย่อยธรรมดา และลิงลูกผสม ในบริเวณที่มีรายงานการผสมข้ามสายพันธุ์และบริเวณใกล้เคียง ระบุชนิดย่อยจากลักษณะทางสัณฐาน สังเกตและทดสอบพฤติกรรมการใช้เครื่องมือหิน เก็บตัวอย่างเลือดและอุจจาระเพื่อใช้วิเคราะห์และระบะชนิดย่อยของลิงจากลักษณะทางพันธุกรรมจากเครื่องหมายพันธุกรรมได้แก่ ไมโทคอนเดรียลดีเอ็นเอ (mtDNA) ยีนบนวายโครโมโซม (*SRY* และ *TSPY*) และ SNP บนโครโมโซมร่างกายด้วยวิธี Restriction Site Associated DNA Sequencing (RADseq) ผลการศึกษาแสดงให้เห็นถึงอิทธิพลของการปฏิวัติกลางและต้นสมัยไพลสโตซีน (EMPT) ต่อการแยกสายวิวัฒนาการของลิงหางยาวชนิดย่อยพม่า โดยการอพยพจากประเทศพม่า/บังคลาเทศลงมาทางตอนใต้ทิศตะวันตกของประเทศไทย การศึกษานี้ยังยืนยันสมมติฐานที่มีมาก่อนหน้าเกี่ยวกับการผสมข้ามสายพันธุ์ที่เกิดจากลิงหางยาวชนิดย่อยพม่าเพศผู้เคลื่อนเข้าสู้ประชากรของลิงหางยาวชนิดย่อยธรรมดา การวิเคราะห์ SNP แสดงให้เห็นถึงระดับของการผสมทางพันธุกรรมในประชากรลิงลูกผสมที่ไม่สมมาตร ที่เกิดลูกผสมเป็นไปในทิศทางเดียว คือ จากลิงหางยาวชนิดย่อยพม่าสู่ลิงหางยาวชนิดย่อยธรรมดา และสามารถตรวจจับประชากรลิงลูกผสมได้ในพื้นที่ที่กว้างกว่าที่เคยมีรายงานมาก่อนหน้านี้ เมื่อวิเคราะห์ความสัมพันธ์ระหว่างชนิดย่อยของลิงที่จำแนกจากลักษณะทางสัณฐานหรือจากลักษณะทางพันธุกรรมกับพฤติกรรมที่เกี่ยวข้องกับการใช้กินที่แบ่งเป็น 4 ระดับ คือ การใช้เครื่องมือในการหาอาหาร การทุบอาหารลงบนหินหรือวัสดุแข็ง การเล่นกับหิน และการไม่แสดงพฤติกรรมที่เกี่ยวกับหิน พบว่า ลิงหางยาวชนิดย่อยพม่าและลิงลูกผสมที่จำแนกโดยใช้ลักษณะทางสัณฐานมีความสัมพันธ์กับพฤติกรรมที่เกี่ยวข้องกับการใช้หินอย่างมีนัยยะสำคัญทางสถิติ ในขณะที่การจำแนกลิงโดยใช้เครื่องหมายพันะุกรรม mtDNA, *SRY* และ *TSPY* ไม่พบความสัมพันธ์กับพฤติกรรมที่เกี่ยวข้องกับการใช้หิน แต่เครื่องหมายพันธุกรรมชนิด SNP ที่บ่งชี้ระดับของการผสมทางพันธุกรรม สามารถบ่งชี้ได้ว่าประชากรลิงหางยาวที่มีระดับของการผสมทางพันธุกรรมของลิงหางยาวชนิดย่อยพม่าที่สูงมีการแสดงออกของพฤติกรรมการใช้เครื่องมือหินและพฤติกรรมการทุบอาหารลงบนหินหรือวัสดุแข็ง ในขณะที่ประชากรลิงหางยาที่มีระดับของการผสมทางพันธุกรรมของลิงหางยาวชนิดย่อยพม่าที่ต่ำ ไม่พบพฤติกรรมที่สัมพันธ์กับหิน ดังนั้นผลการศึกาในครั้งนี้แสดงเป็นนัยยะให้เห็นถึงบทบาทของลักษณะทางพันธุกรรมต่อการเกิดขึ้น การปรากฏ และการพบพฤติกรรมการใช้เครื่องมือในลิงหางยาวชนิดย่อยพม่าและลิงลูกผสม

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Poompat Phadphon : DEGREE OF GENETIC ADMIXTURE IN HYBRID POPULATIONS BETWEEN *Macaca fascicularis fascicularis* AND *M. f. aurea* IN ASSOCIATION WITH STONE TOOL-USE BEHAVIOR. Advisor: Prof. SUCHINDA MALAIVIJITNOND, Ph.D. Co-advisor: Prof. Sreetharan Kanthaswamy, Ph.D.

Burmese long-tailed macaque (*Macaca fascicularis aurea*) is the only Old World monkey that habitually uses stones as tools to access encased food. The natural range of *M. f. aurea* closely contacts to the common long-tailed macaque (*M. f. fascicularis*) in southern Thailand at 8°10'-12°24'N, where the intraspecific hybridization between the two subspecies was reported. While *M. f. aurea* x *M. f. fascicularis* hybrids were expressed a stone-tool use behavior, the behavior has never been seen in the wild or captive *M. f. fascicularis*. It is interesting to understand if the genetic factor plays a role on the emergence of this behavior. This study surveyed the populations of *M. f. aurea*, *M. f. fascicularis* and hybrids at the intraspecific hybrid zone and vicinity, identified morphological subspecies, observed/tested the stone-tool use behaviors, collected blood and fecal samples for genetic analyses, and genetic subspecies identification using mtDNA, Y-chromsome genes (*SRY* and *TSPY*), and autosomal single nucleotide polymorphism (SNP) derived from Restriction Site Associated DNA Sequencing (RADseq) as genetic markers. The findings in this study illustrated the influence of the early-middle Pleistocene transition (EMPT) on the split of *M. f. aurea* lineage through the southward migration from Myanmar/Bangladesh to southwestern Thailand. This study confirmed the previous hypothesis of the male-mediated intraspecific hybridization from *M. f. aurea* to *M. f. fascicularis* populations. In addition, the analysis of RADseq-derived SNPs indicated an asymmetrically (or unidirectionally) genetic introgression of *M. f. aurea* to *M. f. fascicularis* populations that occurred far beyond the previously reported intraspecific hybrid zone. The analysis of an association between the identified morphological subspecies or identified genetic subspecies and the stone-assisted behaviors, that were categorized into 4 levels; stone-tool use behavior, food pounding behavior, stone-play behavior and none, was performed. The statistically significant association between the morphologically identified *M. f. aurea* and hybrids and the complex stone-associated behaviors was detected, while it was no association between the identified genetic subspecies (based on mtDNA, *SRY* and *TSPY*) and the stone-associated behaviors. However, the SNP markers that indicate the degree of genetic admixture in hybrid populations can associate the high degree of *M. f. aurea* ancestry in *M. fascicularis* with stone-play and stone-tool use behaviors and the low degree of *M. f. aurea* ancestry in *M. fascicularis* with non-stone use behavior. Thus, these results imply the possibility of genetic influences on the emergence, the prevalence and the restriction of stone-tool use behavior in *M. f. aurea* and hybrids.

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

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LIST OF ABBREVIATIONS

A&T	Adenine & Thymine
AIDs	Acquired immunodeficiency syndrome
ALP	Ao Lobi plantation
ASP	American Society of Primatologists
BBR	Baan Bor Rae
BIC	Bayesian information criterion
BMS	Ban Mai Somboon school
BNI	Boi Noi island
BNT	Bayin Nyei Temple
bp	base pairs
BRP	Bang Rong pier
BTB	Bang Taboon
BYI	Boi Yai Island
CIA	Check-In andaman pier
CLT	Chong Lat Tai
DNA	Deoxyribose Nucleic Acid
DNP	Department of National Parks, Wildlife, and Plant Conservation of Thailand
EDTA	Ethylenediaminetetraacetic acid
EMPT	Early-Middle Pleistocene Transition
ESS	Effective Sample Size

gDNA	genomic DNA
GPS	Global Positioning Systems
HIV	Human immunodeficiency virus
HPD CI	Highest posterior density credibility interval
HVSI	Hyper variable segment I
IACUC	Institutional Animal Care and Use Committee
JLI	Jarlan Island
kbp	Kilobase pairs
KCS	Khao Chai Son
KKN	Khao Kanab Nam
KKSK	King Kaew Soi Kao
KMD	Klong Mudong
KMI	Khami Island
KNKTK	Khao Noi/Khao Tangkuan
KNY	Khao Na Yak
KRI	Koram Island
KTP	Khao Toh Phyawang
LIL	Lilet
LPI	Lampi Island
LTI	Lanta Island
MCMC	Malkov Chain Monte Carlo
Mfa	*Macaca fascicularis aurea*
Mff	*Macaca fascicularis fascicularis*

MFRC	Mangrove Forest Research Center
ML	Maximum Likelihood
Mm	Macaca mulatta
MRCA	Most Recent Common Ancestor
MSY	Male-specific region of Y chromosome
mtDNA	Mitochondrial DNA
MYA	Million years ago
NaCl	Sodium chloride
NPRCT-CU	National Primate Research Center of Thailand-Chulalongkorn University
NRCT	National Research Council of Thailand
PAR	Pseudoautosomal region
PCR	Polymerase chain reaction
PNI	Panak island
PNN	Piak Nam Noi
PNY	Piak Nam Yai Island
RADseq	Restriction site-associated DNA sequencing
RTL	Relative tail length
RYR	Rayaring island
SDS	Sodium dodecyl sulfate
SIV	Simian immunodeficiency virus
SKP	Sukha Pier
SNP	Single nucleotide polymorphism

SRI	Sirae Island
SRY	Samroiyot National Park
SRY	Sex-determining region Y
SSD	Suan Somdet Prasrinakharin Chumphon
SSP	Suan Somdet Phra Srinakarindra
T&C	Thymine & cytosine
T&T	Thymine & thymine
TKW	Tam khao keeree wong
TLI	Thalu Island
TLP	Thalen pier
TPK	Thamprakayang
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
TSPY	Testis-specific protein, Y-encoded
TST	Ta Sang Tai
WKC	Wat Khao Chong Krachok
WKH	Wat Khuha Phimuk
WKK	Wat khao keaw wichian
WKS	Wat Khuha Sawan
WKT	Wat khao thamon
WKTK	Wat Khao Takiab
WPN	Wat Paknam Pracharangsarith
WSK	Wat Suwan Khuha
WTS	Wat Tham Sue

WWM	World War Museum
YYI	Yao Yai island
ZDK	Zadetkyi

CHAPTER I
GENERAL INTRODUCTION

Burmese long-tailed macaque (*Macaca fascicularis aurea*) is one among ten subspecies of long-tailed macaques (*M. fascicularis*) that are native to the Asian continent (Fooden, 1995). Comparing to common long-tailed macaque (*M. fascicularis fascicularis*) that has the widest distribution range, only next to rhesus macaque (*M. mulatta*), the distribution of *M. f. aurea* is narrow and expanded only from Myanmar to Andaman Sea Coast, southwestern Thailand (Fooden, 1995; San et al., 2011; Bunlungsup et al., 2016). Apart from the three non-human primate species that habitually use stones as tools to forage for foods, i.e., chimpanzees (*Pan troglodytes*) and capuchins (*Sapajus libidinosus* and *S. xanthrosternos*) (Haslam et al., 2009; Wynn et al., 2011), *M. f. aurea* is the fourth one (Malaivijitnond et al., 2007; Gumert et al., 2009). They are the only reported Old World monkey that habitually use stones as tools to process various types of encased foods, e.g., mollusks, crustaceans, and nuts (Gumert et al., 2009; Gumert & Malaivijitnond, 2012; Luncz et al., 2017a). The stone-tool use behavior in *M. f. aurea* was first reported in 1887 while the survey was conducted in Mergui Archipelagos, Myanmar (Carpenter, 1887). This report was neglected, and the behavior was rediscovered in Piak Nam Yai island (9°34' N, 98°28' E), Ranong, Thailand (Malaivijitnond et al., 2007). The spotlight started shedding on them afterward.

Macaca fascicularis aurea has been hypothesized to originate from the ancient hybridization between proto-*M. f. aurea* males and *sinica* species females in Myanmar/Bangladesh (Matsudaira et al., 2018). Their migrations from Myanmar

along the Andaman Sea Coast to southwestern Thailand (Fooden, 1995; San and Hamada, 2011; Bunlungsup et al., 2016) were proposed to have occurred in the late Pleistocene epoch, 21,00 – 9,000 years ago (Bunlungsup et al., 2016). The natural range of *M. f. aurea* closely contacts with that of *M. f. fascicularis* in the southern peninsula of Thailand (Fooden, 1995). These two subspecies can be distinguished based on a cheek hair pattern and head crest. *M. f. aurea* has infrazygomatic cheek hair pattern without a head crest, while *M. f. fascicularis* has transzygomatic cheek hair pattern either with or without a head crest (Fooden, 1995; Bunlungsup et al., 2016). The heterogeneous phenotype, mixed or asymmetric cheek hair pattern, was reported in hybrid macaques at the intraspecific hybrid zone between *M. f. aurea* and *M. f. fascicularis* at 8°10'-12°24'N of Thailand (Fooden, 1995).

Genetic studies based on partial mitochondrial DNA (mtDNA) and Y-chromosome (sex-determining region Y: *SRY* and testis-specific protein, Y-encoded: *TSPY*) genes confirmed the *M. f. aurea* x *M. f. fascicularis* intraspecific hybridization and illustrated the scenario of the hybridization that occurred via male-mediated *M. f. aurea* to *M. f. fascicularis* populations (Bunlungsup et al., 2016). Beyond the information on intraspecific hybridization between *M. f. aurea* and *M. f. fascicularis* and the direction of introgression which had been previously reported, specific knowledge about the degree of genetic admixture between *M. f. aurea* and *M. f. fascicularis* in each hybrid population is still lacking

Apart from the stone-tool use behaviors that were exclusively found in *M. f. aurea*, the hybrids between *M. f. aurea* and *M. f. fascicularis* living on Koram island (12°14′N, 100°0'E) and in Ao Phang-nga National Park (8°10′N, 98°37'E), Thailand have been observed using stone tools (Bunlungsup et al., 2016; Tan, 2017; Luncz et

al., 2019), while there have been no reports in *M. f. fascicularis*, either in the wild or captive animals (Malaivijitnond & Hamada, 2008; Malaivijitnond et al., 2011; Bandini & Tennie, 2018). Among the *M. f. fascicularis* non-stone-tool users, the stone-assisted behaviors, such as food pounding and stone-play, were reported in this subspecies. For food pounding behavior, monkeys perform a direct percussion of food items onto hard substrates, e.g., unmanipulated embedded stones (Tan, 2017). The stone-play behavior is a manipulation of stone in different ways, e.g., rolling and rubbing (Huffman, 1984; Nahallage & Huffman, 2008) to the substrates as seen in Wat Khao Takieb macaques (12°30'N, 99°58'E), Prachuap Khiri Khan, Thailand (Carter et al., 2016). Thus, the stone-play behavior is considered a potential behavioral precursor of stone-tool use behaviors (Huffman & Quiatt, 1986; Hayashi et al., 2005; Leca et al., 2011).

Since only the *M. f. aurea* and *M. f. aurea* x *M. f. fascicularis* hybrids were found using stone as tools to access encased foods, while the *M. f. fascicularis* either play with stones or use them to assist the food pounding, it is interesting to understand if the genetics plays a role on emergence of this behavior. To evaluate the effect of the *M. f. aurea*'s genetic on stone-tool use behavior in *M. fascicularis*, this study surveyed, identified subspecies of *M. fascicularis*, observed/tested the stone-tool use and stone-assisted behaviors, and performed genetic analyses. The study mainly focused on the intraspecific hybrid zone between *M. f. aurea* and *M. f. fascicularis* and its vicinity. For the genetic analyses, partial mtDNA, Y-chromosome genes (*SRY* and *TSPY*), and autosomal single nucleotide polymorphisms (autosomal SNPs) derived from Restriction Site Associated DNA Sequencing (RADseq) were used. This study presented the subspecies status and the genetic structure and composition of *M.*

f. aurea, *M. f. fascicularis*, and *M. f. aurea* x *M. f. fascicularis* hybrids. The study also evaluated the degree of genetic admixture of *M. f. aurea* ancestry in those monkeys in association with stone-tool use behaviors.

Objectives

1. To study the distribution pattern and genetic characteristics of *M. f. aurea*, *M. f. fascicularis*, and *M. f. aurea* x *M. f. fascicularis* hybrids in the southern part of Thailand
2. To observe/test the stone-tool use behaviors in *M. f. aurea*, *M. f. fascicularis*, and *M. f. aurea* x *M. f. fascicularis* hybrids in the southern part of Thailand
3. To evaluate subspecies status and degree of genetic admixture of *M. f. aurea* ancestry in each *M. fascicularis* population in association with stone-tool use behaviors.

CHAPTER II
LITERATURE REVIEW

1. Long-tailed macaques

1.1 General information of long-tailed macaques (*Macaca fascicularis*)

Long-tailed macaques (*M. fascicularis*) are one of the 23 recognized macaque species in the genus *Macaca*, the family Cercopithecidae (Old World monkey) (Li et al., 2015). Based on external male genitalia those 23 macaque speceis are classified into four species groups: *fascicularis*, *silenus-sylvanus*, *sinica*, and *arctoides* (Fooden, 1976). Under this classification, *M. fascicularis* falls into the *fascicularis* species group with the other three macaque species: rhesus macaque (*M. mulatta*), Japanese macaque (*M. fuscata*), and Taiwanese macaque (*M. cyclopis*). If the species group classification is based on the phylogenetic relationship, the macaques are classified into seven species groups: *sylvanus*, *silenus*, *sulawesi*, *sinica*, *arctiodes*, *fascicularis*, and *mulatta* species group (Zinner et al., 2013), where *M. mulatta, M. fuscata, and M. cyclopis* are separated from *M. fascicularis* into their *mulatta* group. Thus, only *M. fascicularis* remains in *fascicularis* species group.

M. fascicularis is a commonly used nonhuman primate model for biomedical research (Bonhomme et al., 2009). The natural distribution of *M. fascicularis* ranges from 21ºN to 10ºS and from 92ºE to 126ºE (Fooden, 1995). Their distribution covers mainland Southeast Asia, shallow-water islands on Sunda Shelf, and deep-water islands extending from southernmost Bangladesh, Myanmar, Thailand, Laos, Vietnam, Cambodia, Malaysia, Indonesia, Timor, the Philippines, and Nicobar Islands (Fooden, 1995). Though a large number of *M. fascicularis* are found in Mauritius

islands in southeast Africa, they were not native to the islands and were likely to be introduced from Indonesia probably in the 16th century (Sussman & Tattersall, 1986; Tosi & Coke, 2007).

The characters of *M. fascicularis* vary geographically (Fooden, 1995). Generally, their dorsal pelage colors vary from buffy to yellowish gray to golden brown to reddish-brown to dark brown to blackish, which is similar among adult, subadult, and juvenile males and females. Adult males are typically larger than adult females, with around 13% longer crown-rump length and 49% heavier body mass. The relative tail length (RTL; tail length/crown-rump length x 100) is similar in adult males and females by 117 ± 14.5 % on average (Fooden, 1995).

As a result of widespread distribution and geographically variable morphological characters, *M. fascicularis* can be classified into 10 subspecies: common long-tailed macaque (*M. f. fascicularis*), Burmese long-tailed macaques (*M. f. aurea*), dark-crowned long-tailed macaques (*M. f. atriceps*), Con Song long-tailed macaque (*M. f. condorensis*), Karimunjawa long-tailed macaque (*M. f. karimondjawae*), Nicobar long-tailed macaque (*M. f. umbrosa*), Simeulue long-tailed macaque (*M. f. fusca*), Lasia long-tailed macaque (*M. f. lasiae*), Maratua long-tailed macaque (*M. f. tua*), and Philippine long-tailed macaque (*M. f. philippinensis*) (Fooden, 1995). Among the 10 subspecies, *M. f. fascicularis*, *M. f. aurea*, and *M. f. philippinensis* occupy most of the distribution areas of *M. fascicularis*, while others distribute only in small islands (Figure 2.1; Fooden 1995). In Thailand, three subspecies, i.e., *M. f. fascicularis, M. f aurea, and M. f. atriceps* were reported (Fooden, 1995). *M. f. fascicularis* is the most common subspecies and can be found almost throughout the country (Malaivijitnond & Hamada, 2008; Bunlungsup et al., 2016), while *M. f. aurea* has been reported only

in Ranong province (Malaivijitnond & Hamada, 2008; Bunlungsup et al., 2016), and *M. f. atriceps* is restricted on Koh Khram Yai (12º 40' - 12º 43' N, 100 º 4' - 100º 48' E), a small island in the gulf of Thailand (Fooden, 1995). Although there have been no further reports of *M. f. atriceps* in Thailand after Fooden reported in 1995 (Fooden, 1995), *M. f. fascicularis* and *M. f. aurea* have been intensively studied within the past 15 years (Malaivijitnond & Hamada, 2008; Bunlungsup et al., 2016; Bunlungsup, 2017a).

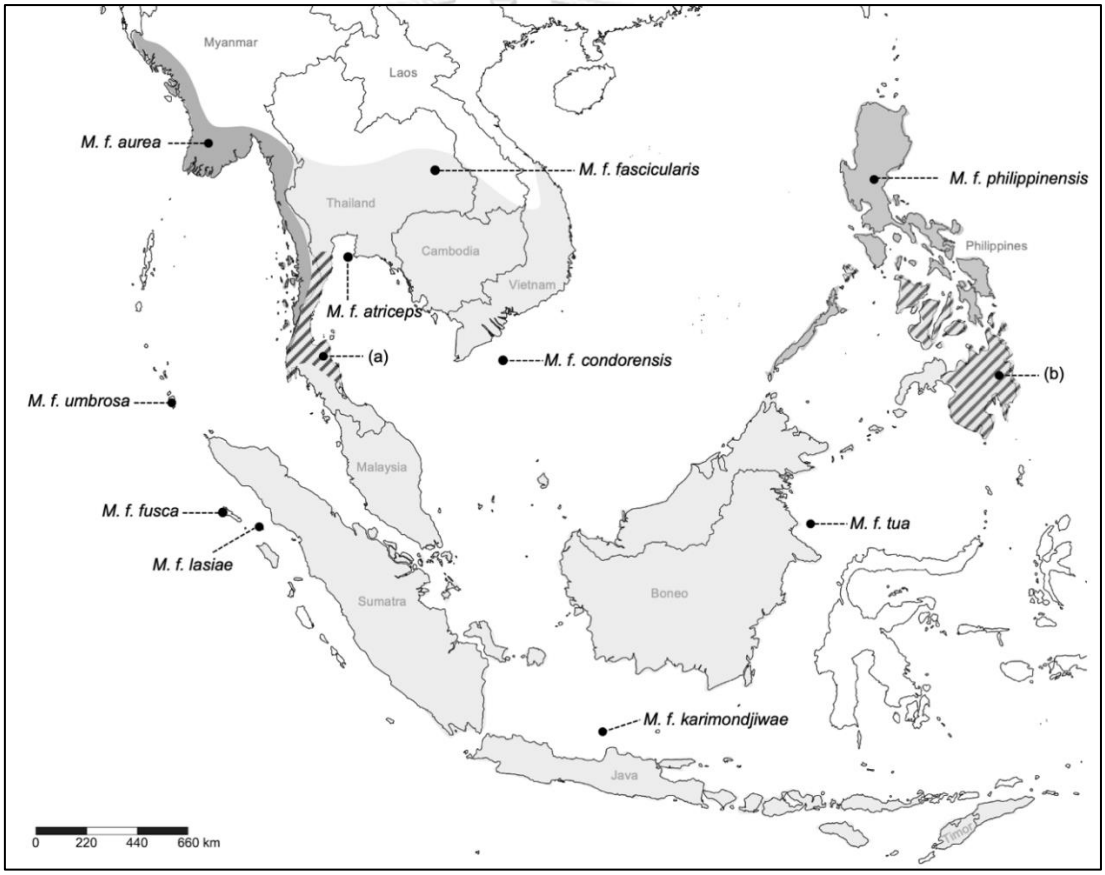


Figure 2.1 The distributions of the ten subspecies of *Macaca fascicularis*. Hatched pattern indicates subspecific contact zones between *M. f. aurea* and *M. f. fascicularis* (a) and between *M. f. fascicularis* and *M. f. philippinensis* (b).

1.2 Burmese long-tailed macaque (*Macaca fascicularis aurea*)

Given a common name of the Burmese long-tailed macaque, *M. f. aurea* distributes mainly in Myanmar. Indeed, their natural distribution spans from southeasternmost Bangladesh, along the coast of the Bay of Bengal and Andaman Sea of Myanmar, to the Mergui Archipelago and a small area of southwestern Thailand (Figure 2.1; Fooden 1995; San and Hamada, 2011; Bunlungsup et al., 2016). In Bangladesh, although over 200 individuals used to be reported, only a few (<10) individuals remained in the coastal mangrove near the border between Bangladesh and Myanmar (Hasan & Feeroz, 2010; Kabir & Ahsan, 2012), and on July 27th, 2022 an extinction of this subspecies in Bagladesh was announced (https://www.tbsnews.net/environment/nature/extinction-alert-bangladesh-bids farewell-long-tailed-macaques-465874). Considering the distribution in Thailand, *M. f. aurea* populations seem to be restricted in Ranong province and closely contact to *M. f. fascicularis* (Malaivijitnond & Hamada, 2008), where the *M. f. aurea* x *M. f. fascicularis* intraspecific (intersubspecific) hybridization was reported (Fooden, 1995; Bunlungsup et al., 2016). Compared to the *M. f. fascicularis* subspecies, the *M. f. aurea* subspecies has a darker dorsal pelage color, has no head crest, and has an infrazygomatic pattern of the lateral facial crest hairs: the crest occurs near but inferior to the mandibular region and terminates superiorly in a whorl shape on the cheek, and hairs of the temporal region are posteriorly directed from an eye to an ear (Figure 2.2a, Fooden, 1995; Bunglungsup et al., 2016).

Bunlungsup et al. (2016) analyzed the phylogenetic relationship of *M. f. aurea*, *M. f. fascicularis*, and *M. mulatta* based on partial mtDNA sequences and indicated a closer genetic relationship between *M. f. fascicularis* and *M. mulatta* than between *M.*

f. fascicularis and the conspecific *M. f. aurea*, which diverged first in the phylogenetic tree. Later, Matsudaira et al. (2018) showed the phylogenetic relationship based on the whole mitochondrial genome of *M. f. aurea*. They found that the mitochondrial genome of *M. f. aurea* was clustered with the *sinica* species group instead of *fascicularis*/*mulatta* species group, whereas Y-chromosome (*SRY* and *TSPY* gene) sequence was yet grouped with *M. fascicularis.* The incongruence between mitochondrial and Y-chromosome phylogenetic trees was proposed to result from the ancient introgression of male proto-*M. f. aurea* into the *sinica* species group and hybridized in approximately 2.5 - 0.98 MYA in Myanmar-Bangladesh areas. This introgression probably led to the nuclear swamping of which the nuclear genome of the *sinica* species group had been replaced by those of proto-*M. f. aurea* resulted in the morphological characteristics and Y-chromosome of *M. f. aurea*, while the mitochondrial genome orginated from the *sinica* species group in *M. f. aurea* (Matsudaira et al., 2018). However, the comparison of admixture patterns on X chromosome and autosomes of *M. f. aurea* failed to support the nuclear swamping hypothesis that the X chromosomes should be less affected by introgression than autosomes (Osada et al., 2021). Nonetheless, the substantial admixture between *M. f. aurea* and the s*inica* species group was detected, supporting the origin of *M. f. aurea* from the ancient hybridization with the *sinica* species group (Osada et al., 2021).

1.3 Common long-tailed macaque (*Macaca fascicularis fascicularis*)

Among the 10 subspecies of *M. fascicularis*, *M. f. fascicularis* occupies the widest area ranging from Indochinese peninsula (Thailand, Laos, Vietnam, Cambodia), Malay peninsula, Sumatra, Borneo, Java, lesser Sunda, Timor, and the

south-central Philippines (Figure 2.1, Fooden, 1995). Unlike *M. f. aurea*, *M. f. fascicularis* has a brighter pelage color, the lateral facial crest hairs sweep upward from near the angle of the jaw to the lateral margin of the crown, the hairs of the temporal region are anteriorly directed, so-called transzygomatic pattern, and the head crest is either present or absent (Figure 2.2a, Fooden, 1995; Bunglungsup et al., 2016).

Based on the mtDNA (Tosi & Coke, 2007; Liedigk et al., 2015; Bunlungsup, 2017a) and Y-chromosome gene phylogenetic analyses (Bunlungsup, 2017a; Rovie-Ryan et al., 2021), *M. f. fascicularis* could be divided into two major groups; insular and continental. The insular group consists of the populations distributed in south Sumatra, Java, Borneo, Timor, and the Philippines. In contrast, the continental group comprises the Indochina region (including Thailand, Vietnam, and Cambodia), the Malay peninsula, and North Sumatra. According to the comprehensive genetic analyses of samples collected throughout Thailand and the vicinity, Bunlungsup et al. (2017a) identified four more distinctive subclades within the continental clade: Indochina, Vietnam, Sundaic Thai Gulf, and Sundaic Andaman Sea Coast (including southernmost Thailand, Malaysia, and northern Sumatra). Although *M. f. fascicularis* populations in Sundaic Thai Gulf and Sundaic Andaman Sea Coast subclades live closer to each other in the south of the Isthmus of Kra (10° 30' N), the Sundaic Andaman Sea Coast subclade is genetically closer to Vietnam and Indochina subclades living above the Isthmus of Kra. This has been proposed to have resulted from the expansion and emigration of *M. f. fascicularis* from southern Indochina around eastern Thailand, southern Cambodia, and Vietnam to the southernmost part of Thailand, Malay peninsula, and northern Sumatra via the Sunda Shelf when the sea

level was low in the glacial period (Bunlungsup et al., 2017a). Sundaic Thai Gulf subclade was found to be distinct from other subclades (Bunlungsup et al., 2017a), which sometimes was recognized as the third major clade of *M. f. fascicularis* (Matsudaira et al., 2018).

2. Intraspecific hybridization between *M. f. aurea* and *M. f. fascicularis*

M. f. aurea and *M. f. fascicularis* are distributed close to each other in mainland southeast Asia and are separated by the Tenasserim hill lying along the border between Myanmar and Thailand (Fooden, 1995, San & Hamada, 2011). These two subspecies can mainly be distinguished using lateral facial crest hair patterns: tranzygomatic and infrazygomatic (Figure 2.2a, Fooden, 1995; Bunlungsup et al., 2016). In southern Thailand where the two subspecies lived overlapping, the heterogeneity of facial crest-hair-patterns of mixed or asymmetric (transzygomatic and infrazygomatic on different side of the cheek) type was reported and the intraspecific (or intersubspecific) hybridization was proposed at 8°10'-12°24'N (Figure 2.2b, Fooden, 1995).

Bunlungsup et al. (2016) confirmed the intersubspecific hybridization between *M. f. aurea* and *M. f. fascicularis* by showing the incongruence of mtDNA and Y-chromosome DNA haplotypes in those populations. The hybrids carried the mtDNA haplotype of *M. f. fascicularis* and the Y-chromosome haplotype of *M. f. aurea*. Unlike those of *M. f. fascicularis* x *M. mulatta* hybridization scenario, *M. f. aurea* x *M. f. fascicularis* hybrids possessed Y-chromosome haplotypes of either *M. mulatta*/*M. f. fascicularis* or *M. f. aurea*. Two possibilities of this hybridization event were proposed: i) the hybridization occurred recently, or ii) neither *M. mulatta*/*M. f.*

fascicularis nor *M. f. aurea* Y-chromosome haplotypes had more advantages to be selected over the other (Bunlungsup et al., 2016). Besides, no hybrid populations having the mtDNA haplotypes of *M. f. aurea* were found, indicating only unidirectional male-mediated hybridization from *M. f. aurea* to *M. f. fascicularis* population has occurred. Considering the different mtDNA haplotypes and localities, the *M. f. aurea* x *M. f. fascicularis* hybrid populations were divided into Indochinese and Sundaic, which carried Indochinese and Sundaic mtDNA haplotypes of *M. f. fascicularis*, respectively. The Indochinese group lived on the mainland and island of the Thai gulf above the Isthmus of Kra, and the Sundaic group occupied below the Isthmus of Kra along the Andaman Sea Coast. These hybrid groups probably occurred in two different hybridization events where the Tenasserim hill is the natural barrier separating the two subspecies. The first event was that *M. f. aurea* migrated southward along Andaman Sea Coast and hybridized with Sundaic *M. f. fascicularis*, and the second event was that some of *M. f. aurea* moved north eastward to Thai gulf and hybridized with Indochinese *M. f. fascicularis* (Bunlungsup et al., 2016).

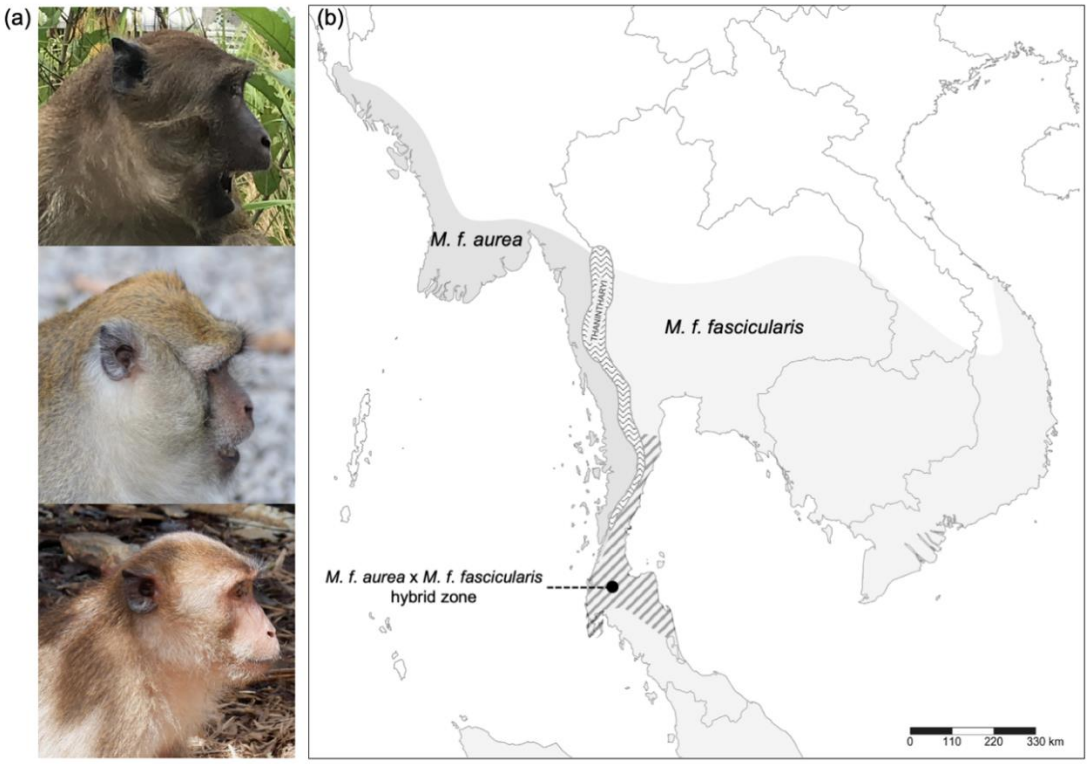


Figure 2.2 Morphological characteristics and distributions of *M. f. aurea*, *M. f. fascicularis* and *M. f. aurea* x *M. f. fascicularis* hybrid. (a) Infrazygomatic (top), transzygomatic (middle), and mixed cheek pair patterns (bottom) in *M. f. aurea*, *M. f. fascicularis*, and hybrids between *M. f. aurea* and *M. f. fascicularis*, respectively. (b) Distributions of *M. f. aurea*, *M. f. fascicularis*, and the proposed hybrid zone between *M. f aurea* and *M. f. fascicularis* in Thailand.

3. Tool use

3.1 Definition of tool use behaviors

The definition of the tool-use behavior has been debated over the past years (Beck, 1980; St Amant & Horton, 2008; Shumaker et al., 2011; Hunt et al., 2013). Although definitions of "tool use" are slightly different, it can affect the consideration of behaviors as tool use. The explicit definition applied in the tool use study is essential. Shumaker et al. (2011) thoroughly considered, integrated prior definitions, and gave the revision, with a clear explanation, based on widespread-used Beck's original definition (1980) as:

"*The external employment of an unattached or manipulable attached environmental object to alter more efficiently the form, position, or condition of another object, another organism, or the user itself, when the users hold and directly manipulates the tool during or prior to use and is responsible for the proper and effective orientation of the tool*."

According to the above definition, large unmanipulated anvils in pounding behavior, i.e., nut cracking, are not considered tools. Nonetheless, the unmanipulated anvils are called proto-tools to acknowledge their functional similarity to moved or manipulated anvils. They further stated that the manipulation of only the *object of change* (altered object), but not the *agent of change* (tool), is proto-tool use, e.g., the herring gull picks up the mussel (altered object) and drops it on the stones (Shumaker et al., 2011). The definition of "to alter" implies goal-directedness in using objects. It allows distinguishing tool use from object manipulation or object play, which Shumaker et al. (2011) define as "holding or directly manipulating unattached or

attached environmental objects with no evident proximate (immediate and tangible) purpose.

Beck's original definition of tool use has influenced animal tool-use studies for decades (St. Amant & Horton, 2008). Giving more updated information, Shumaker et al. (2011) revised Beck's original definition, which was employed in this study.

3.2 Factors play roles in tool use prevalence

After the tool-use behavioral studies were boosted, the factors underpinning the behavior's presence or absence have become an interesting subject of research (Boesch et al., 1994; McGrew et al., 1997). Ecological, genetic, and cultural factors have been under discussion (Boesch et al., 1994; McGrew et al., 1997; Laland & Janik, 2006; Sargeant et al., 2006; Bacher et al., 2010).

Undoubtedly, the ecological environment affects animals' tool behaviors (Byrne, 2007; Hansell & Ruxton, 2008; Sanz & Morgan, 2013). It provides the materials required for the behaviors. In bottlenose dolphins (*Tursiops* sp.), a sponging behavior, which dolphins use a sponge to cover their rostra while foraging, was significantly correlated with ecological factors of sponge coverage and water depth (Sargeant et al., 2006). Nonetheless, the absence of nut-cracking and termite fishing in some chimpanzee populations could not be explained by the limitation of environmental factors required for the behaviors (Boesch et al., 1994; McGrew et al., 1997; Whiten et al., 1999). Hansell & Ruxton (2008) stated that the occurrence of animal tool uses resulted from the ecological contexts in which using tools was more advantageous (so-called the lack-of-utility hypothesis), while Hunt et al. (2013) disagreed with this idea. They argued that the rarity of tool use in the animal kingdom did not harmonize

with ample opportunity for tool use to evolve (the excess-of-opportunity problem). For example, the termites and wood-boring grubs were expected to be exploited by many animal species because they are widely distributed food sources with extremely high nutrition (Hunt et al., 2013). However, only chimpanzees (Whiten et al., 1999) and two species of birds (New Caledonian crows, *Corvus moneduloides* and woodpecker finches, *Cactospihza pallida*) habitually use tools to exploit them, which contradicted the lack-of-utility hypothesis (Hunt et al., 2013).

Langergraber et al. (2011) indicated a significantly positive correlation between genetic and behavioral dissimilarity among chimpanzee populations living in Africa. Naïve woodpecker finches and New Caledonian crows developed tool-use behaviors to retrieve food from tree holes of crevices without any demonstrations or social input from conspecifics (Tebbich et al., 2001; Kenward et al., 2006). These findings illustrated at least partial genetic predisposition influences on animal tool-use behaviors. Most tool use behaviors in birds and primates are considered the flexible or creative tool uses that they use multiple tools to solve multiple problems, which are recognized as a cognitive demanding (Call, 2013; Hunt et al., 2013). Later, the positive correlation between manipulation complexity and brain size/cognitive test performance in primates was indicated (Heldstab et al., 2016). Genetics influences on cognitive abilities and development (humans) (Hill et al., 2014; Lam et al., 2017; Mollon et al., 2021) and tool-use performances (chimpanzees) (Hopkins et al., 2015; Hopkins et al., 2016) were reported.

The patchy distribution of tool-use behaviors, e.g., termite fishing and nut cracking among chimpanzee populations that were not correlated with ecological contexts, were considered cultural variations (Boesh et al., 1994; McGrew et al.,

1997; Whiten et al., 1999). Typically, culture is considered a group-specific behavior shared by members within a community that are socially learned and transmitted, and independent of ecological and genetic factors (Boesch et al., 1994; Laland & Hoppitt, 2003). The explicit example of cultural transmission was reported in chimpanzees. O'Malley et al. (2012) reported the spread of ant-fishing in the chimpanzee community of Kazekela, Gombe National Park, which immigrant chimpanzees introduced from the habitually ant-fishing chimpanzee community of Mitumba, Gombe National Park (O'Malley et al., 2012). Although excluding an ecological factor is practical, a genetic influence on behavioral variation cannot be easily ruled out. Genetic influences have been reported to contribute to motor skill acquisition and the rate of learning in human twins (Fox et al., 1996).

3.3 Stone tool-use in *M. fascicularis*

Apart from chimpanzees (*Pan troglodytes*) and capuchins (*Sapajus libidinosus* and *S. Xanthrosternos*) (Haslam et al., 2009; Wynn et al., 2011), *M. fascicularis* is another species of non-human primates that habitually use stones as tools (Malaivijitnond et al., 2007; Gumert et al., 2009). They are the only Old-World monkeys found to perform this behavior for daily foraging (Luncz et al., 2019).

Stone tool-use behavior in *M. fascicularis* was first negligibly reported over 120 years ago (Carpenter, 1887), which later got the spotlight after rediscovery in 2007 in Thailand (Malaivijitnond et al., 2007). The stone tool-use behavior in *M. fascicularis* was found only in *M. f. aurea* subspecies and *M. f. aurea* x *M. f. fascicularis* hybrids (Gumert et al., 2009; 2019; Bunlungsup et al., 2016). In other subspecies, though the researchers tried to train them in captivity or intensively surveyed throughout their

distribution range, no stone tool-use behavior has been discovered, e.g., *M. f. fascicualris* (Malaivijitnond & Hamada, 2008; Banidi & tennie, 2018; Gumert et al., 2019) and *M. f. umbrosa* (Pal et al., 2018).

Stone tool-use behavior in *M. fascicularis* can be classified into two major types: axe hammering and pound hammering (Malaivijitnond et al., 2007; Gumert et al., 2009). Macaques perform axe hammering to crack attached food items, especially oysters, by holding hand-sized stones with a finger-to-thump passive palm grip (a precision grip). They apply pound hammering with unattached food items, e.g., marine snails, gastropods, and nuts, by gripping stone tools with a power-finger/active palm grip (Malaivijitnond et al., 2007; Gumert et al., 2009; Gumert & Malaivijitnond, 2012; Lunzc et al., 2017a).

M. fascicularis's stone-tool use seems to be restricted on islands and coastal regions with intertidal, mangrove habitats (Gumert & Malaivijitnond, 2012; Luncz et al., 2017b; Tan, 2017; Gumert et al., 2018; Luncz et al., 2019), reflecting the crucial ecological factors on stone tool-use prevalence. Like other non-human primate stone-tool users, *M. fascicularis* develop stone tool-use behavior through tool use-related object manipulation before percussive behavior and successful tool use (Tan, 2017). Social influences (or cultural influences) have also been illustrated in the development of the behavior as young macaques preferentially interact with the closely socially-associated skillful tool users. This interaction bias can provide a better opportunity to socially learn about tool use during the developmental period (Tan et al., 2018). Later, Gumert et al. (2019) studied the stone-tool use behavior in one *M. f. aurea* x *M. f. fascicularis* hybrid population living on Koram Island, Khao Sam Roi Yot National Park, Thailand. They categorized this macaque population into two groups based on

their lateral facial crest patterns: hybrid-like macaques and *M. f. fascicularis*-like macaques. The hybrid-like macaques showed a significantly higher prevalence of tool users than in *M. f. fascicularis*-like macaques. Nonetheless, neither ecological nor social factors were sufficient to explain the variation of tool-use prevalence between these two different phenotype groups of *M. f. aurea* x *M. f. fascicularis* hybrids because they lived together in the same ecological and cultural conditions (Gumert et al., 2019). It underlined the possibility of genetically inherited disposition that may play a role on the emergence, prevalence, and restriction of this behavior in *M. f. aurea*.

3.4. Stone-assisted behavior

Stone handling or stone manipulation has been proposed as a behavioral precursor of stone-tool use (Huffman & Quiatt, 1986; Leca et al., 2011). The ontogeny of stone tool use in *M. fascicularis* indicated object manipulation, especially stone, before the development of successful stone-tool use (Tan, 2017). The stone manipulation has been reported across phylogenetically closed species in the *fascicularis*/*mulatta* species group (*M. fascicularis*; Pelletier et al., 2017; Carter et al., 2016, *M. mulatta, M. fuscata* and *M. cyclopis*; Huffman, 1984; Nahallage & Huffman, 2012; Nahallage et al., 2016), implying the shared propensity for stone manipulation across this *species* group (Nahallage & Huffman, 2012; Pelltier et al., 2017). This behavior has no immediate and tangible function. It does not directly contribute to feeding or reproductive fitness, which is considered object play behavior or stone play behavior in this case (Nahallage et al., 2016). Thirty years of stone play studies in *M. fuscata* by Huffman's team (Leca et al., 2011) illustrated an accumulation of diversity

and complexity of the behavioral patterns over time which highlighted the opportunity of functional uses of stones to emerge, e.g., an integration of stone manipulation with foraging activity (Leca et al., 2011; Nahallage et al., 2016). This supported the consideration of stone play/stone manipulation as a behavioral precursor of stone-tool use (Huffman & Quiatt, 1986; Hayashi et al., 2005; Leca et al., 2011; Tan, 2017).

Tan et al. (2017) reported that among the non-stone-tool using *M. fascicularis* of Koram's population, some carried out direct percussion of food items on hard substrate, and some rubbed food items on the substrate but never percussed them (Tan, 2017). A test of nut-cracking behavior in naïve chimpanzees illustrated that a chimpanzee, who failed to crack open a nut, rarely manipulated stone and was lack of percussive actions during object manipulation bouts (Hayashi et al., 2005). Apart from the difficulty of multiple objects combined, the scarcity of percussive action and stone manipulation was considered the critical factors that obstructed the emergence of stone-tool use in chimpanzees (Hayashi et al., 2005).

4. Genetic markers for population and hybridization analysis

4.1 Mitochondrial DNA

mtDNA is an extranuclear DNA found in mitochondria. It is a small, circular DNA molecule typically, in animals, ranging from 15 to 20 kilobase pairs (kbp) (Gray, 2013). In vertebrates, mtDNA comprises 13 genes encoding for protein in oxidative phosphorylation, two ribosomal RNA genes, 22 transfer RNA genes, and the non-coding region containing controlling elements for replication and transcription, the so-called control region (Harrison, 1989; Boore, 1999; Taanman, 1999). According to the active degradation model and/or simple dilution model (Sato

& Sato, 2013), mtDNA is strictly inherited from maternal parent to offspring in the most sexually reproducing organisms (Taanman, 1999; Gray, 2013). Because of this maternal inheritance, together with the higher rate of mutation compared to the nuclear DNA, as well as a rare occurrence of recombination, mtDNA has long become a genetic marker in studies of population and evolutionary biology, especially for tracing maternal genealogy (Harrison, 1989; Taanman 1999; Gray 2013). In the genetic studies of macaques, the whole and partial mtDNA sequences were analyzed (Liedigk et al., 2015; Bunlungsup et al., 2017a; Matsudaira et al., 2018). Among the parts of mtDNA, the control region has the highest rate of mutation, making it as the mutational hotspot (Stoneking, 2000), and therefore, becomes a common target of genetic analyses in closely related species-level and intraspecific population-level (Marmi et al., 2004; Sun et al., 2010; Yao et al., 2013; Bunlungsup et al., 2016).

4.2 Sex-determining region Y and testis-specific protein, Y-encoded genes

X and Y chromosomes are the sex chromosomes of all mammals and many other animal species (Ross & Blackmon, 2016) and are believed to evolve from once autosomal chromosomes, in which the Y differentiated from the X by stratification that hampered recombination with the X and followed by degeneration (Hughes & Rozen, 2012; Bachtrog, 2013). Compared to the humans and chimpanzees, the Y chromosome of *M. mulatta*, as a representative of Old World monkeys, is much smaller and comprises approximately 11 megabases (Hughes et al., 2012). Apart from the single pseudoautosomal region (PAR), the whole *M. mulatta*'s Y chromosome segment has no meiotic recombination with the X, so-called the male-specific region

of Y chromosome (MSY), and therefore inherits only in patriline (Hughes et al., 2012).

The sex-determining region Y (*SRY*) and testis-specific protein, Y-encoded (*TSPY*) are genes on MSY encoding a protein that initiates the cascade processes of testis formation (Kashimada & Koopman, 2010) and spermatogenesis-involved protein in testicular tissue (Schnieder et al., 1996), respectively. As they are inherited paternally, *SRY* and *TSPY* have been used as paternal genetic markers in evolutionary and phylogeographic studies of mammal species (Moreira, 2002; Nishida et al., 2003), including macaques (Tosi et al., 2000, 2002; Ogawa & Vallender, 2014; Bunlungsup, 2017a). Bunlungsup et al. (2016; 2017a) analyzed *SRY* and *TSPY* sequences in *M. mulatta*, *M. f. fascicularis*, and *M. f. aurea*. Although there are a few polymorphic sites, the 2.3 kbp segments of *TSPY* sequences could provide informative results to distinguish the three taxa and clarify the male-mediated hybridization/introgression between *M. mulatta* - *M. f. fascicularis* and *M. f. aurea* – *M. f. fascicularis*. Within 686 bp of *SRY* segments, it comprises two polymorphic sites that are enough for the preliminary screening before the *TSPY* gene will be conducted.

4.3 Single nucleotide polymorphisms (SNPs)

Although the mtDNA and Y-chromosome gene sequences can be used to differentiate the two subspecies of *M. fascicularis*; *M. f. fascicularis,* and *M. f. aurea*, and indicate the hybrid, it cannot refer to the level of genetic admixture of the ancestral species in the hybrid individuals. SNP is a single base variation on the DNA sequence that occurs approximately 300 to 1,000 bp throughout the genome (Morin et al., 2004). According to SNPs' abundance and genome-wide coverage, it becomes the

marker of choice in molecular ecology, evolution, and conservation (Morin et al., 2004; Brumfield et al., 2016). Although any of four possible nucleotide bases can be present at each position, SNPs are usually bi-allelic (Vignal et al., 2002). Compared to a multi-allelic microsatellite, bi-allelic SNPs might be less informative in some kinds of studies, e.g., parentage analyses. Nonetheless, this limitation can be overcome by using more loci (Brumfield et al., 2003). In addition, SNP markers can provide more accurate genetic distance-based parameters, e.g., F_{ST} population differentiation and inbreeding coefficient F (Vignal et al., 2002; Brumfield et al., 2003; Morin et al., 2004). The use of microsatellites could raise some concerns in population genetic inferences; that is, the mutation patterns of microsatellites can vary among loci which can be problematic in model-based inferences (Putman & Carbone, 2014; Brumfield et al., 2016). In the review of Putman & Carbone (2014), they stated some major problems with using microsatellites in model-based clustering as in STRUCTURE analysis (Pritchard et al., 2000). This includes inference of weak structure, a confounding effect of incomplete lineage sorting, and a large number of loci that are needed to accurately identify population structure, admixture, or hybrids, and the null alleles. Besides, the coefficient similarity between repeated runs of STRUCTURE indicated significantly higher SNP values than microsatellites (Garke et al., 2012). Homoplasy, an allele at a given locus that becomes identical by state not by descent, that can occur more often in microsatellites, is another issue commonly cited as a drawback in the use of microsatellites as genetic markers for population analysis (Morin et al., 2004; Putman & Carbone 2014).

RADseq is the restriction-enzyme-based next-generation sequencing method for high-throughput SNP discovery and genotyping (Baird et al., 2008; Davey et al.,

2011; Andrews et al., 2016). It represents the genome's subset, which enables the identification of thousands to tens of thousands of SNPs within both coding and non-coding regions throughout the genome (Davey & Blaxter, 2010; Davey et al., 2011). For the RADseq, the genomic DNA is digested with a restriction enzyme. The enzyme used can variate the number of loci produced, e.g., a common-cutter enzyme cuts more frequently while a rare-cutter enzyme generates more DNA fragments sequenced (Davey et al., 2011). The DNA fragments are then ligated with adaptors containing forward primer and sequencing primer sites, e.g., Illumina sequencing primers, as well as the in-line barcodes (short unique sequences for sample identification). The adapter-ligated fragments are multiplexed, mechanically sheared, and added to a second adapter. The fragments ligated with both adaptors are PCR amplified. This means that only fragments with the restriction sites are amplified and sequenced. The post-sequencing analyses will initiate with de-multiplexing and filtering reads based on sequence quality. The reads will be aligned to a reference genome or assembled *de novo* if a reference genome is not available. Genotypes can be called, then followed by a filtering step for SNP quality control (Baird et al., 2008; Andrews et al., 2016).

RADseq is the cost-effective technique to discover and genotype thousands of SNP markers across many samples in a single experiment whether the prior genomic information of the studied organism is available (Baird et al., 2008; Andrews et al., 2016). It has been used in many ecological, evolutionary, and population genetic studies, especially in non-model organisms (Wagner et al., 2013; Ford et al., 2015; Chen et al., 2020; Ito et al., 2020). RADseq data also enables the development of a smaller SNP panel but is still highly informative and could be used with other cost-

saving methods to escalate sample size and require less quality DNA (Karam et al., 2015; Stetz et al., 2016; Guppy et al., 2020).

Thus, in this study, the mtDNA, Y chromosome genes (*SRY* and *TSPY*), and RADseq SNP makers were used to indicate the *M. f. aurea*, *M. f. fascicularis*, and *M. f. fascicularis* x *M. f. aurea* hybrid populations, and the association between each population of macaques and stone-assisted behaviors (stone tool-use, food pounding, stone-play, and none) was analyzed.

CHAPTER III
INTRASPECIFIC HYBRIDIZATION AND DIVERGENCE TIME ESTIMATION OF *Macaca fascicularis aurea*

Introduction

Macaca fascicularis aurea, the only stone tool user among the Old-World monkeys (Luncz et al., 2019), distributes mainly in Myanmar, Mergui Archipelago, and the Andaman Sea Coast, southwestern Thailand (Figure 3.1). The divergence of *M. f. aurea* in mainland Myanmar from those in the Mergui Archipelago and Thailand (Bunlungsup et al., 2016; Matsudaira et al., 2018), using mtDNA analysis, supported the hypothesis that *M. f. aurea* originated in Myanmar/Bangladesh then migrated southeastwardly to southwestern Thailand (Fooden, 1995; San and Hamada, 2011). The natural range of *M. f. aurea* closely contacts with *M. f. fascicularis*, the most widely distributed *M. fascicualris* subspecies in Thailand (Fooden, 1995). According to the comprehensive genetic analyses of *M. f. fasciularis* throughout Thailand, Bunlungsup et al. (2017a) identified three distinct subclades: Indochina, Sundaic Thai Gulf, and Sundaic Andaman Sea Coast. While *M. f. fascicularis* located below the Isthmus of Kra in the Sundaic region (Hughes et al., 2011) possess *M. f. fascicularis* Y-chromosome haplotypes, those located above the Isthmus of Kra in Indochina region possess *M. mulatta* Y-chromosome haplotypes (Bunlungsup et al., 2017a).

As mentioned in Chapter II, *M. f. aurea* and *M. f. fascicularis* can simply be distinguished based on a cheek hair pattern and a head crest, and the hybrid between the two subspecies has the heterogeneous, mixed or asymmetric cheek hair pattern. The hybrids identified based on these morphological characters were reported in the

peninsula part of Thailand at 8°10'-12°24'N (Fooden, 1995). Later, the partial mtDNA and Y-chromosome DNA analyses confirmed the intraspecific hybridization between the two subspecies in the region (Bunlungsup et al., 2016). The hybridization process was proposed through male *M. f. aurea* migration into *M. f. fascicularis* populations (Bunlungsup et al., 2016; Matsudaira et al., 2018; Osada et al., 2021). The hybrids can be classified into two groups based on their localities and mtDNA and Y-chromosome analyses: Indochinese and Sundaic (Bunlungsup et al., 2016). However, the Y-chromosome gene (*SRY* and *TSPY*) analyses in the previous study (Bunlungsup et al. 2016) partially supported the hypothesis because none of the Sundaic hybrids was included in the analysis.

The early-middle Pleistocene Transition (EMPT), ranging from 1.4 – 0.4 MYA (Head & Gibbard, 2015), is well-known for the increase in glacial-interglacial cycle interval and the high amplitude of climate oscillations and sea-level changes (Clark et al., 2006; Head & Gibbard, 2015). This transition led to the increase in average global ice volume (Willeit et al., 2019) and strengthened the aridity across the globe, including central and northern Asia (Head & Gibbard, 2015). The effect of EMPT has been reported to contribute to terrestrial biota composition (Head & Gibbard, 2015) and genetic and evolutionary consequences, e.g., diversification of animal species (Hewitt, 2004; Othman et al., 2020; Ghane-Ameleh et al., 2021). Based on the previous mtDNA sequence analysis indicating that *M. f. aurea* migrated from Myanmar to Thailand during the late Pleistocene epoch of 21,00 – 9,000 years ago, it is interesting to know if the EMPT had an effect on the distribution of *M. f. aurea*. Thus, this study surveyed the *M. fascicularis* populations in Thailand, mainly focusing on the intraspecific hybrid zone between *M. f. aurea* and *M. f. fascicularis*,

performed mtDNA and Y-chromosome gene sequence analyses, and estimated divergence time which illustrated the potential influence of the EMPT on lineage split of *M. f. aurea*.

Methods

Ethical statement

The permit for research and sample collection in Thailand was approved by the National Research Council of Thailand (NRCT) and the Department of National Parks, Wildlife, and Plant Conservation of Thailand (DNP). The Institutional Animal Care and Use Committee (IACUC) of the National Primate Research Center of Thailand-Chulalongkorn University (NPRCT-CU) approved the experimental protocols of this study (Protocol Review no. 1975007). The research adhered to the American Society of Primatologists (ASP) Principles for the Ethical Treatment of Non-Human Primates.

Morphospecies identification and sample collections

The survey, morphospecies identification, and biological specimen collection were done in 25 populations of *M. fascicularis* in Thailand (Table 3.1, Figure 3.1), focusing mainly on the intersubspecific hybrid zone between *M. f. fascicularis* and *M. f. aurea* (8°10'-12°24'N; Fooden, 1995; Bunlungsup et al., 2016). If an access to monkeys was possible, the subspecies of *M. fascicularis* populations was first identified based on their cheek hair patterns as described above.

Blood or fecal samples from those 25 populations were collected in this study (see Table 3. 1). Using the trapping and sampling techniques described in

Malaivijinod et al. (2008), the blood samples were centrifuged at 1000 xg for 10 min, and the buffy coat was harvested for DNA extraction. Genomic DNA (gDNA) was extracted using the Gentra Puregene Blood kit (QIAGEN Inc., Hilden, Germany) following the manufacturer's protocol. The fecal samples were non-invasively collected from defecated specimens for gastrointestinal cells. The feces' surface was swabbed using cotton buds and stirred in 1 mL lysis solution (0.5% (w/v) SDS, 100 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0 and 10 mM NaCl) (Hayashi & Kawamoto, 2006). To maximize the number of gastrointestinal cells harvested, these steps were repeated 2-3 times per sample before storing the solution at room temperature until DNA extraction. The gDNA was extracted from the solution using the QIAamp DNA/Fast DNA Stool Mini kit (QIAGEN Inc., Hilden, Germany) following the manufacturer's protocol. The gDNA samples of WKTK were obtained from the DNA Bank of the NPRCT-CU, Saraburi, Thailand, which were extracted from blood samples using the standard phenol-chloroform method (Sambrook et al., 1989). Sequences of mtDNA and Y-chromosome genes of 13, 8, and 4 populations of *M. f. fascicularis*, *M. f. aurea*, and *M. f. fascicularis* x *M. f. aurea* hybrids were also accessed from the previous reports (Bunlungsup et al. 2016, Bunlungsup et al., 2017a).

Table 3.1 Locality, GPS coordinates, type of specimen, and subspecies identification based on phenotype and genotype of *M. fascicularis* populations included in this study. *Mfa*, *Mff*, and hybrid are *M. f. aurea*, *M. f. fasicularis*, and *M. f. aurea* x *M. f. fasicularis* hybrid, respectively.

Locality	GPS (N, E)	Type of specimen	Phenotype	mtDNA	*SRY*	Genotype	Note
1. Bayin Nyei Temple (BNT)	16°58', 97°29'	Blood	*Mfa*	*Mfa*	*Mfa*	*Mfa*	Bunlungsup et al. (2016)
2. Ta Sang Tai (TST)	15°56', 99°57'	Blood	*Mff*	*Mff*	*Mm*	*Mff*	This study
3. Bang Taboon (BTB)	13°15', 99°56'	Feces	*Mff*	*Mff*	*Mm*	*Mff*	This study
4. Wat khao thamon (WKT)	13°02', 99°57'	Blood	*Mff*	*Mff*	*Mm*	*Mff*	Bunlungsup et al. (2016)
5. Wat Khao Takiab (WKTK)	12°30', 99°58'	Blood	Hybrid	*Mff*	*Mm/ Mff*	*Mff*	This study
6. Koram Island (KRI)	12°14', 100°00'	Feces	Hybrid	*Mff*	*Mm/ Mfa*	Hybrid	Bunlungsup et al. (2016)
7. Samroiyot National Park (SRY)	12°07', 99°57'	Feces	Hybrid	*Mff*	*Mm/ Mfa*	Hybrid	Bunlungsup et al. (2016)
8. Wat Khao Chong Krachok (WKC)	11°48', 99°48'	Blood	Hybrid	*Mff*	*Mm/ Mfa*	Hybrid	Bunlungsup et al. (2016)
9. Lampi Island (LPI)	10°54', 98°12'	Feces	*Mfa*	*Mfa*	*Mfa*	*Mfa*	Bunlungsup et al. (2016)
10. Ban Mai Somboon school (BMS)	10°51', 99°13'	Feces	*Mfa*	*Mff*	*Mfa*	Hybrid	Bunlungsup et al. (2016)
11. Jarlan Island (JLI)	10°25', 97°56'	Feces	*Mfa*	*Mfa*	-	-	Bunlungsup et al. (2016)
12. Thamprakayang (TPK)	10°19', 98°45'	Blood	Hybrid	*Mfa*	*Mfa*	*Mfa*	This study
13. World War Museum (WWM)	10°10', 98°43'	Blood	*Mfa*	*Mfa*	*Mfa*	*Mfa*	This study
14. Zadetkyi (ZDK)	9°58', 98°11'	Feces	*Mfa*	*Mfa*	*Mfa*	*Mfa*	Bunlungsup et al. (2016)
15. Wat Paknam Pracharangsarith (WPN)	9°57', 98°35'	Blood	*Mfa*	*Mfa*	*Mfa*	*Mfa*	Bunlungsup et al. (2016)
16. Suan Somdet Prasrinakharin Chumphon (SSD)	9°56', 99°02'	Blood	*Mff*	*Mff*	*Mm*	*Mff*	Bunlungsup et al.(2017a)
17. Mangrove Forest Research Center (MFRC)	9°52', 98°36'	Blood	*Mfa*	*Mfa*	*Mfa*	*Mfa*	Bunlungsup et al. (2016)
18. Piak Nam Yai Island (PNY)	9°35', 98°28'	Feces	*Mfa*	*Mfa*	*Mfa*	*Mfa*	Bunlungsup et al. (2016)
19. Piak Nam Noi (PNN)	9° 35', 98°28'	Feces	-	*Mfa*	-	-	This study
20. Lilet (LIL)	9°13', 99°14'	Feces	*Mff*	*Mff*	-	-	This study
21. Tam khao keeree wong (TKW)	9°12', 99°39'	Feces	*Mff*	*Mff*	-	-	Bunlungsup et al.(2017a)
22. Khao Na Yak (KNY)	8°35', 98°13'	Feces	Hybrid	*Mff*	-	-	This study
23. Check-In andaman pier (CIA)	8°33', 98°13'	Feces	Hybrid	*Mff*	*Mff/ Mfa*	Hybrid	This study
24. Suan Somdet Phra Srinakarindra (SSP)	8˚26', 98˚31'	Feces	*Mff*	*Mff*	*Mff*	*Mff*	Ei Than (2017)
25. Wat Suwan Khuha (WSK)	8°25', 98°28'	Blood	*Mff*	*Mff*	*Mff*	*Mff*	Bunlungsup et al. (2016)

Table 3.1 continue.

Locality	GPS (N, E)	Type of specimen	Phenotype	mtDNA	*SRY*	Genotype	Note
26. Thalu Island (TLI)	8°18', 98°28'	Feces	-	*Mff*	*Mff*	*Mff*	This study
27. Rayaring island (RYR)	8°17', 98°29'	Feces	-	*Mff*	*Mff*	*Mff*	This study
28. Chong Lat Tai (CLT)	8°15', 98°37'	Feces	-	*Mff*	-	-	This study
29. Wat Khao Keaw Wichian (WKK)	8°12', 100°05'	Blood	*Mff*	*Mff*	*Mff*	*Mff*	Bunlungsup et al.(2017a)
30. Panak island (PNI)	8°12', 98°29'	Feces	*Mff*	*Mff*	*Mff*	*Mff*	This study
31. Boi Noi island (BNI)	8°10', 98°32'	Feces	Hybrid	*Mff*	*Mff*	*Mff*	This study
32. Ao Lobi plantation (ALP)	8°10', 98°37'	Feces	Hybrid	*Mff*	-	-	This study
33. Thalen pier (TLP)	8°08', 98°44'	Feces	*Mff*	*Mff*	*Mff*	*Mff*	This study
34. Wat Tham Sue (WTS)	8°07', 98°55'	Feces	*Mff*	*Mff*	-	-	Bunlungsup et al.(2017a)
35. Khami Island (KMI)	8°07', 98°40'	Feces	Hybrid	*Mff*	-	-	This study
36. Boi Yai Island (BYI)	8°07', 98°33'	Feces	Hybrid	*Mff*	*Mff*	*Mff*	This study
37. Yao Yai island (YYI)	8°07', 98°31'	Feces	Hybrid	*Mff*	*Mff*	*Mff*	This study
38. Sukha Pier (SKP)	8°06', 98°35'	Feces	Hybrid	*Mff*	*Mff*	*Mff*	This study
39. Khao Kanab Nam (KKN)	8°04', 98°55'	Feces	*Mff*	*Mff*	-	-	This study
40. Bang Rong pier (BRP)	8°02', 98°24'	Feces	Hybrid	*Mff*	*Mfa*	Hybrid	This study
41. King Kaew Soi Kao (KKSK)	7°54', 98°24'	Feces	Hybrid	*Mff*	*Mff/ Mfa*	Hybrid	This study
42. Sirae Island (SRI)	7°53', 98°25'	Feces	Hybrid	*Mff*	*Mff/ Mfa*	Hybrid	Ei Than (2017)
43. Baan Bor Rae (BBR)	7°50', 98°23'	Feces	Hybrid	*Mff*	*Mff/ Mfa*	Hybrid	This study
44. Klong Mudong (KMD)	7°50', 98°22'	Feces	Hybrid	*Mff*	*Mfa*	Hybrid	This study
45. Wat Khuha Sawan (WKS)	7°37', 100°04'	Feces	*Mff*	*Mff*	-	-	Bunlungsup et al.(2017a)
46. Lanta Island (LTI)	7°28', 99°05'	Feces	*Mff*	*Mff*	-	-	Bunlungsup et al.(2017a)
47. Khao Chai Son (KCS)	7°27', 100°07'	Blood	*Mff*	*Mff*	-	-	Bunlungsup et al.(2017a)
48. Khao Noi/Khao Tangkuan (KNKTK)	7°12', 100°35'	Blood	*Mff*	*Mff*	*Mff*	*Mff*	Bunlungsup et al. (2016)
49. Khao Toh Phyawang (KTP)	6°37', 100°03'	Feces	*Mff*	*Mff*	*Mff*	*Mff*	Bunlungsup et al.(2017a)
50. Wat Khuha Phimuk (WKH)	6°31', 101°13'	Blood	*Mff*	*Mff*	*Mff*	*Mff*	Bunlungsup et al.(2017a)

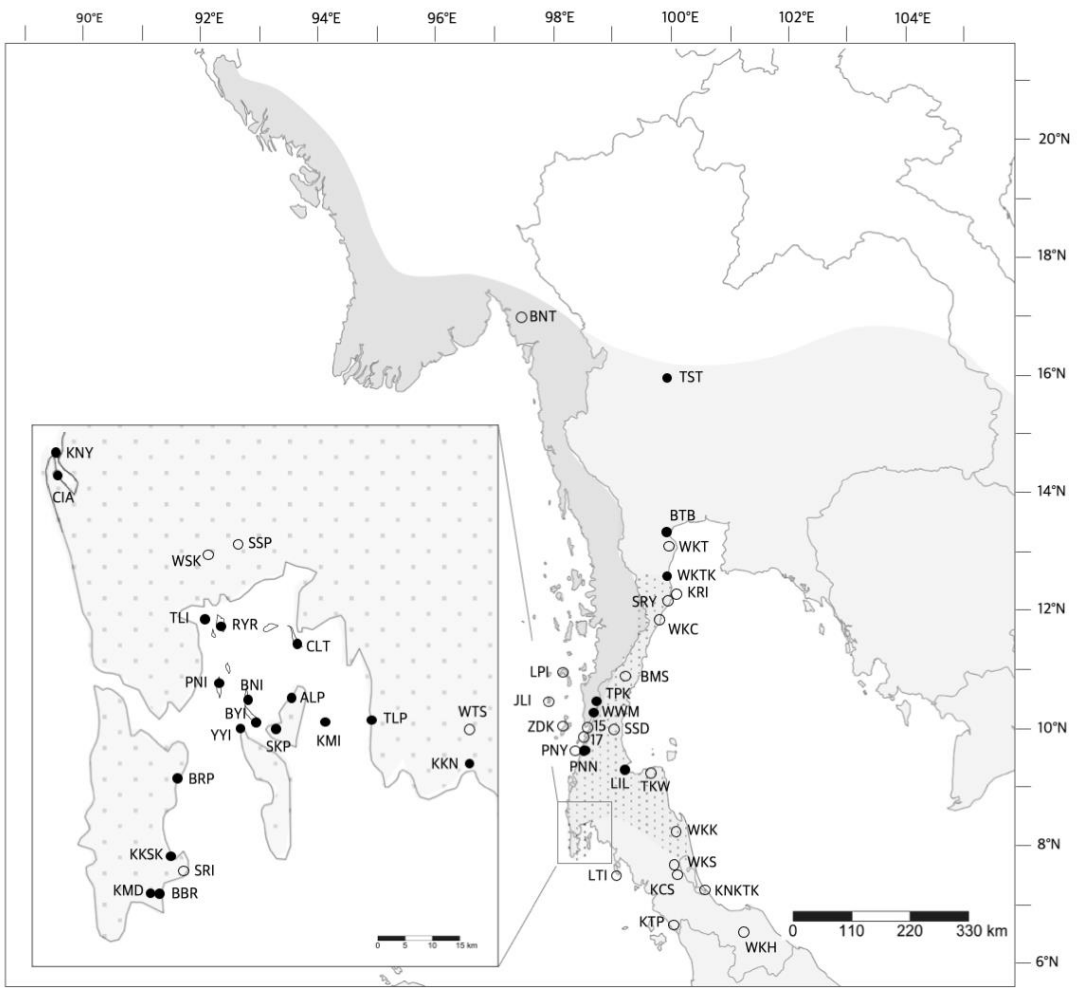


Figure 3.1. The distribution ranges of *Macaca fascicularis aurea* (dark gray), *M. f. fascicularis* (gray shade), and hybrid (dotted pattern) are based on the studies of Fooden (1995) and Bunlungsup et al. (2016). Solid and open circles indicate *M. fascicularis* populations analyzed in this study and in the previous reports (Bunlungsup et al. 2016, Bunlungsup et al., 2017a), respectively. Three to four-letter codes are the names of populations corresponding to those in Table 3.1. On the map, Nos. 15 and 17 indicate WPN and MFRC populations, respectively.

PCR amplification and sequencing of partial mtDNA and Y-chromosome genes

The mtDNA was amplified using HVS-F (5'-CCGCCCACTCAGCCAATTCC TGTTCT-3') and HVS-R (5'CCCGTGATCCATCGAGATGTCTT-3') primers (Bunlungsup et al., 2016), of which the product size was 835 bp covering the hypervariable segment I (HVSI) of the D-loop region, tRNA proline, tRNA threonine, and cytochrome b. The amplification was carried out at 94°C for 1 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, and ended up with 72°C for 5 min for final elongation. For Y-chromosome genes, two regions were amplified and sequenced: *SRY* and *TSPY*. The 216 base pairs (bp) of the partial *SRY* gene was amplified using SRY-FN (5'-TCGCAGCCTCCTTGTTTTTGA-3') and SRY-RN (5'-TCATGGGTCGCTTCACTTTATCC-3') primers (Matsudaira et al., unpublished data) with 94°C for 1 min, 40 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 1 min, followed by 72°C for 5 min. Within 216 bp of *SRY* sequences, two polymorphic sites (nos. 42 and 132 of *SRY* Refseq of *M. mulatta* (NM 001032836.1)) were acquired and used to identify *M. f. aurea* (T&C), *M. f. fascicularis* (A&T) and *M. mulatta* (T&T), respectively. After the *M. f. aurea-SRY* haplotypes were identified, the *TSPY* gene of those monkeys was subsequently amplified using three primer pairs (TSPY-A/TSR1012, TSF566/TSR1676, TSF1383/TSPY-5R) following Bunlungsup et al. (2016) and the product sizes were 1012, 1110, and 855 bp, respectively. The PCR cycles of *TSPY* amplifications were 40 cycles of 94°C for 25 sec, 45 sec of 66°C for TSPY-A/TSR1012, 64°C for TSF566/TSR1676, 66°C for TSF1383/TSPY-5R, and 72 °C for 3 min, followed by a final 72°C for 7 min. PCR mixtures of mtDNA, *SRY*, and *TSPY* amplifications

contained 0.5 U ExTaq DNA Polymerase (Takara Bio Inc., Shiga, Japan), 0.3 mM each primer and 50–100 ng DNA template in the manufacturer's buffer.

The PCR products were run on 2% (w/v) SYBR Safe stained agarose gel-TAE electrophoresis and visualized under the Nucleic acid Bioimaging Instrument (NαBI) blue illuminator (Neo Science Co., Ltd., South Korea). The PCR amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega Corporation, USA) following the manufacturer's protocol before submitting to Macrogen, Inc. (South Korea) for sequencing with the same primer sets.

Phylogenetic tree analyses

DNA sequences were trimmed and aligned using BioEdit 7.2 (Hall, 1999). As for mtDNA, the phylogenetic trees were constructed with the Maximum Likelihood (ML) and Bayesian Inference method. One-hundred fourteen sequences of mtDNA at 674-bp size were analyzed. The GenBank-retrieved Chinese *M. mulatta* sequence (LC093173) and *M. sylvanus* sequence (NC002764) were included in the analysis as outgroups. The Bayesian information criterion (BIC) using jModelTest 2.1.10 (Posada, 2008) was applied to find the best substitution model for the data. The ML tree was constructed under HKY+G+I model with 1000 bootstraps in MEGA X (Kumar et al., 2018). The Bayesian tree was constructed under the same model by using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The analysis was run for 15,000,000 generations, and parameters were sampled every 500 generations. The convergence of the MCMC runs was checked in Tracer 1.5 (Rambaut & Drummond, 2009) with the trace plot and over 200 effective sample size (ESS) values for all parameters. The first 25% of data 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 tree was visualized in FigTree 1.3.1. (Rambaut, 2010). The p-distance UPGMA was performed to cluster *SRY* sequences in MEGA X.

TSPY haplotype network construction

Two *TSPY* sequences of TPK, WWM, CIA, and SRI and one *TSPY* sequence of BRP were successfully amplified. Although *SRY* sequences of SRI were obtained from Ei Than (2017), the *TPSY* sequences were performed in this study. The *TSPY* sequences of BNT, LPI, ZDK, KRI, SRY, WKC, BMS, WPN, MFRC, and PNY, which were reported with the haplotypes of *M. f. aurea* in Bunlungsup et al. (2016), were included in the analysis. The *TPSY* sequences of Chinese *M. mulatta*, Indochinese *M. f. fascicularis*, and Sundaic *M. f. fascicularis* retrieved from GenBank were included in the analysis as reference sequences. The 2039 bp of 37 *TSPY* sequences were used to construct the median-joining haplotype network using PopART 1.7 (Leigh et al., 2015).

Divergence time estimation

Divergence times of mtDNA sequences were estimated using BEAST 2.4.2 (Bouckaert et al., 2014). Divergence time estimation was run with HKY+I+G substitution model as used in the mtDNA phylogenetic tree constructions. The relaxed log normal clock was used with the coalescent Bayesian skyline as a tree prior. The divergence time of African and Asian macaques at 5.5 MYA with normal distribution of 1 MYA 95% credibility interval (Alba et al., 2014) was used as a calibration node. The run was performed with 50,000,000 MCMC generations, and the parameters were

collected every 1,000 generations. Convergence and ESS values were checked using Tracer 1.5. The first 10% of data was discarded, and the consensus tree was generated using TreeAnnotator and visualized in FigTree 1.3.1.

Results

Morphospecies identification

Among 25 populations of *M. fascicularis* newly investigated in this study, their morphological characters could be clearly observed, and subspecies status could be identified in 21 populations. The remaining 4 populations (PNN, RYR, CLT, TLI) that lived on the islands were either not habituated and fled away after the human appearance or did not show up during the survey. Regarding the cheek hair and head crest patterns, TST, BTB, LIL, PNI, KKN, and TLP monkeys had a transzygomatic cheek hair pattern and head crest as seen in *M. f. fascicularis*. The WWM monkeys had an infrazygomatic cheek hair pattern and no head crest as seen in *M. f. aurea*. Fourteen populations of WKTK, TPK, KNY, CIA, BNI, ALP, KMI, BYI, YYI, SKP, BRP, KKSK, BBR, and KMD had mixed characters of *M. f. fascicularis* x *M. f. aurea*, that is, a mixed cheek hair pattern. The morphospecies identification of each population is inferred further in the phylogenetic analyses appended below.

Phylogenetic analyses of the mtDNA and *SRY* genes

The ML and Bayesian phylogenetic trees of mtDNA showed a similar topology; therefore, only the Bayesian tree was used in this study (Figure 3.2). The tree indicated the divergence of *M. f. aurea* clade from *M. mulatta*/*M. f. fascicularis* clade before the divergence of the *M. mulatta* and *M. f. fascicularis* clades. Within the

M. f. aurea clade, a similar result as observed in the study of Bunlungsup et al. (2016) was appeared. The mainland Myanmar *M. f. aurea* population (BNT) was separated first from Mergui Archipelago/Thailand subclade. Within the Mergui Archipelago/Thailand subclade, *M. f. aurea* TPK, WWM, and WPN populations grouped with posterior probability/bootstrap support values of 0.62/90 and separated from subclade of other *M. f. aurea* Thailand (MFRC, PNY, PNN) and Mergui Archipelago (LPI, ZDK, JLI) populations that group together with 0.53/- of the support values. Nonetheless, these subclades of *M. f. aurea* Mergui Archipelago/Thailand clade were polytomies in the ML tree. As for *M. f. fascicularis* clade, TST, BTB, and WKTK populations were grouped with the WKT, WKC, KRI, SRY, BMS populations located above the Isthmus of Kra (10° 15' N, 99° 30' E) in Indochina region (Hughes et al., 2011), and this was called as Indochinese subclade in Bunlungsup et al. (2017a). In the Sundaic region, samples of all *M. f. fascicularis* populations collected in this study were grouped together with SSD, TKW, WSK, SRI, WKK, WKS, KCS, and KNKTK populations as one subclade, except one sample of TLP (TLP25) that was grouped with *M. f. fascicularis* SSP, WTS, LTI, and WKH populations in another subclade. These two Sundaic subclades were named Sundaic Thai Gulf and Sundaic Andaman Sea Coast in Bunlungsup et al. (2017a).

Regarding the analysis of the *SRY* sequences, *M. f. fascicularis* TST and BTB populations were grouped with *M. mulatta* and other *M. f. fascicularis* populations located north of the Isthmus of Kra and the *M. f. fascicularis* SSD population that located slightly south of the Isthmus of Kra (Figure 3.3), this was named as *M. mulatta*/Indochinese *M. f. fascicularis* clade in Bunlungsup et al. (2016, 2017a). The *SRY* sequences of RYR, PNI, TLI, BNI, TLP, BYI, YYI, and SKP–populations

indicated the *M. f. fascicularis SRY* type and were grouped with Sundaic *M. f. fascicularis* clade. The *SRY* sequences of TPK, WWM, KMD, BRP, and BBR populations clustered within *M. f. aurea* clade (Figure 3.3), while mtDNA sequences from the latter three populations were grouped with *M. f. fascicularis* clade (Figure 3.2) Similar to those of *M. f. aurea* x *M. f. fascicularis* KRI, WKC, SRY and SRI hybrid populations reported previously (Bunlungsup et al., 2016; Ei Than, 2017), the coexistence of both *SRY* haplotypes of *M. f. fascicularis* and *M. f. aurea* was found in the CIA and KKSK hybrid populations. The contradiction between mtDNA and *SRY* results and the coexistence of both *SRY* haplotypes of *M. f. fascicularis* and *M. f. aurea* within a population indicated a genotypically hybrid status (see Table 3.1).

Contradiction of subspecies identification based on morphospecies and mtDNA/*SRY* sequence analyses

The contradiction of subspecies identification based on phenotypes (cheek hair pattern and head crest) and genotypes (mtDNA and *SRY* sequence analyses) was found in six populations (Table 3.1). WKTK, BNI, BYI, YYI, and SKP monkeys were morphologically identified as hybrids, while their mtDNA and *SRY* haplotypes indicated the *M. f. fascicularis* (Figure 3.2, 3.3). BMS population was morphologically *M. f. aurea* but possessed mtDNA haplotypes of *M. f. fascicularis* and *SRY* haplotypes of *M. f. aurea*. Morphologically hybrid TPK possessed both mtDNA and *SRY* sequences of *M. f. aurea*.

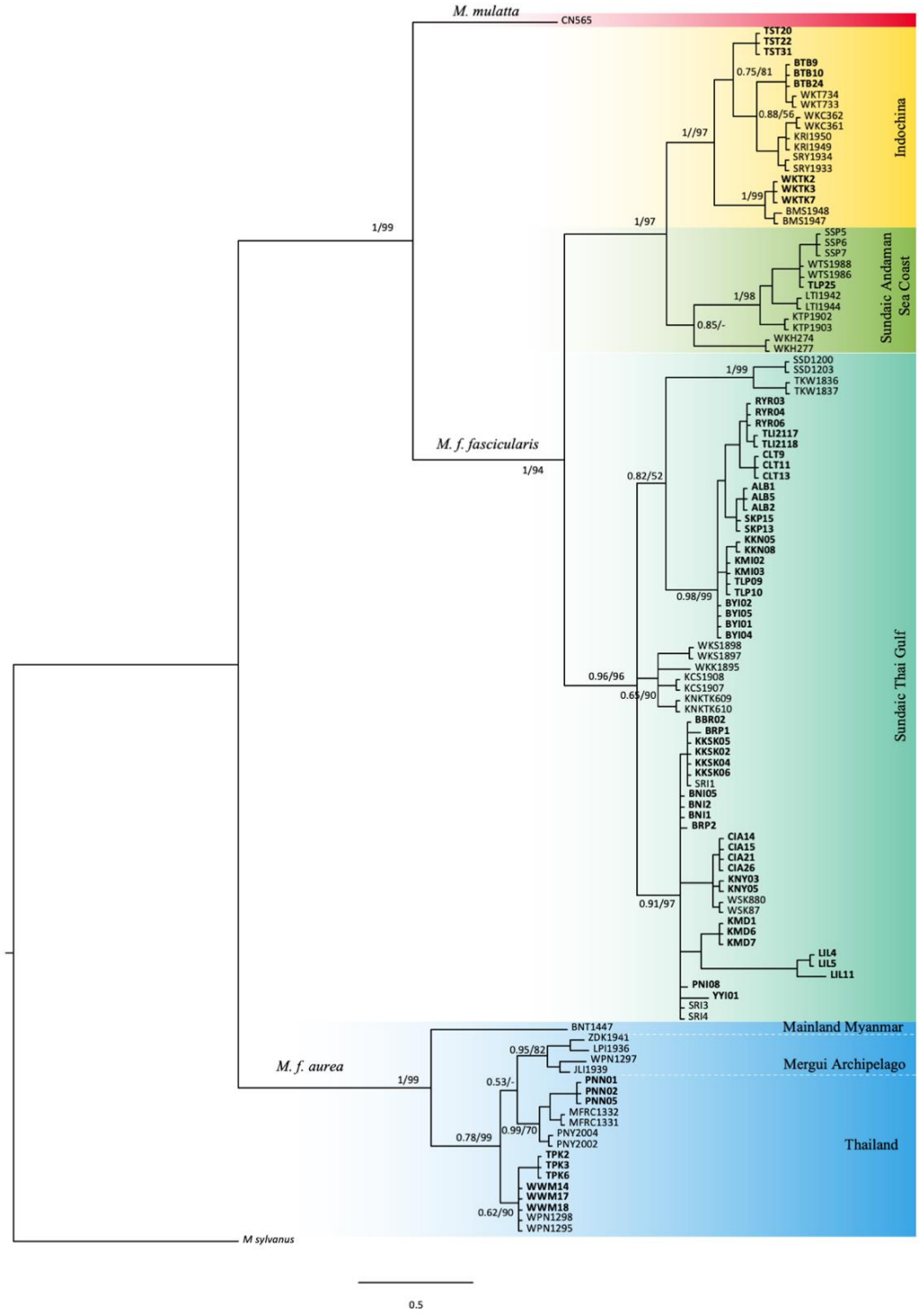


Figure 3.2 Bayesian phylogenetic tree based on 674 bp of the mtDNA gene. The tree's three to four-letter codes correspond to those in Table 3.1. Bold letters indicate the samples that were analyzed in this study. The numbers on each branch refer to the posterior probability/bootstrap values.

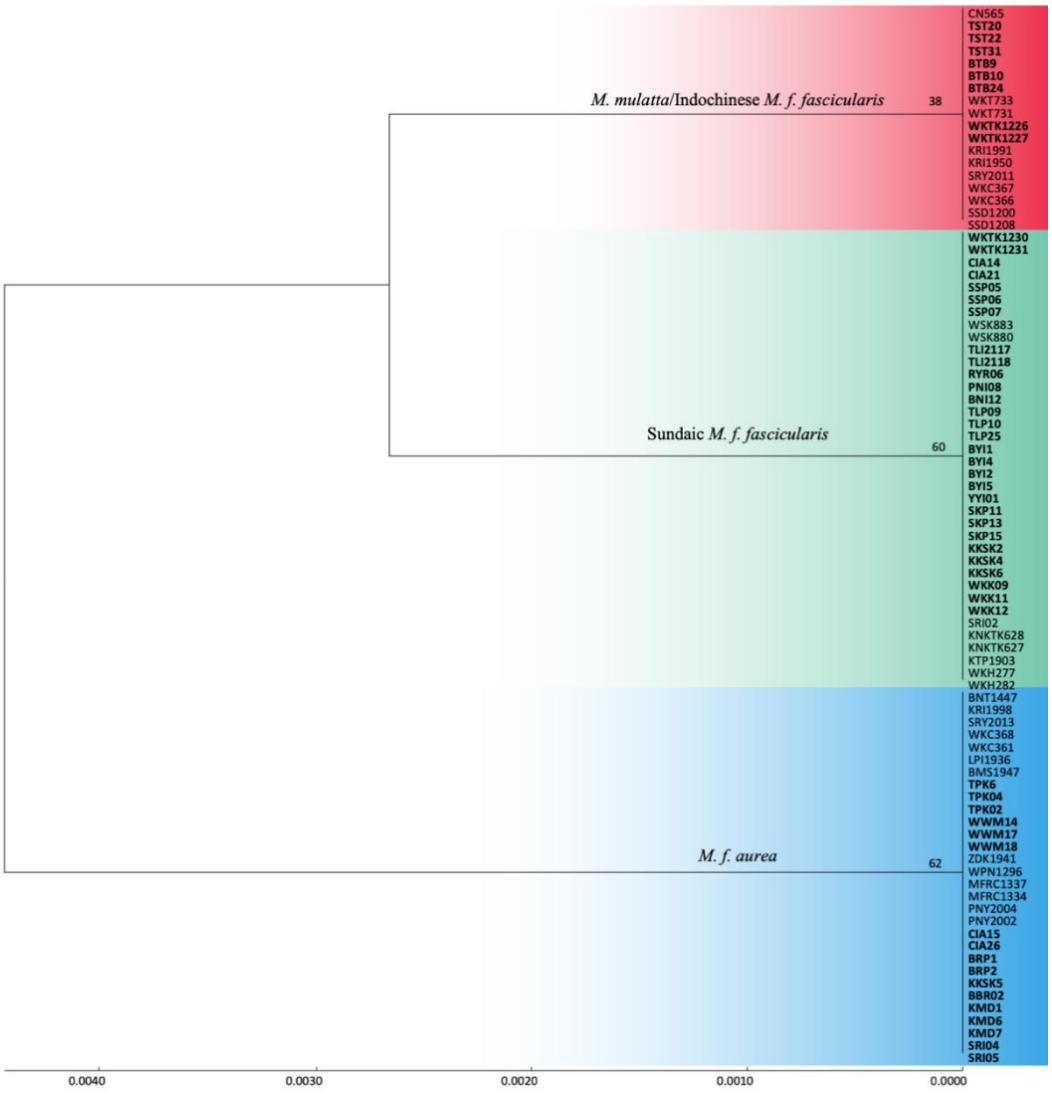


Figure 3.3 UPGMA tree based on 184 bp of the *SRY* gene. Three to four-letter codes on the tree correspond to those in Table 3.1. Bold letters indicate the samples that were analyzed in this study. The numbers on each branch refer to bootstrap values.

TSPY haplotype network

There were three *TSPY* haplotypes of *M. f. aurea* (Figure 3.4); 1, 2, and 3. Samples of Mergui Archipelago *M. f aurea* (LPI, ZDK), Thailand *M. f. aurea* (TPK, WWM, MFRC, PNY), and Sundaic hybrid (CIA, BRP, SRI) shared the same *TSPY* Haplotype 3. Interestingly, one Thailand *M. f. aurea*, WPN, was in Haplotype 2 as seen in SRY and WKC Indochinese *M. f. aurea* x *M. f. fascicularis* hybrids. One individual of Indochinese *M. f. aurea* x *M. f. fascicularis* hybrid from KRI shared the same *TSPY* Haplotype 1 with mainland Myanmar *M. f. aurea* (BNT).

M. mulatta and Indochinese *M. f. fascicularis* shared in a single haplotype. Sundaic *M. f. fascicularis* possessed one haplotype. *M. mulatta*/Indochinese *M. f. fascicularis* and Sundaic *M. f. fascicularis TPSY* haplotypes were more closely related and distinct from the three *TPSY* haplotypes of *M. f. aurea*.

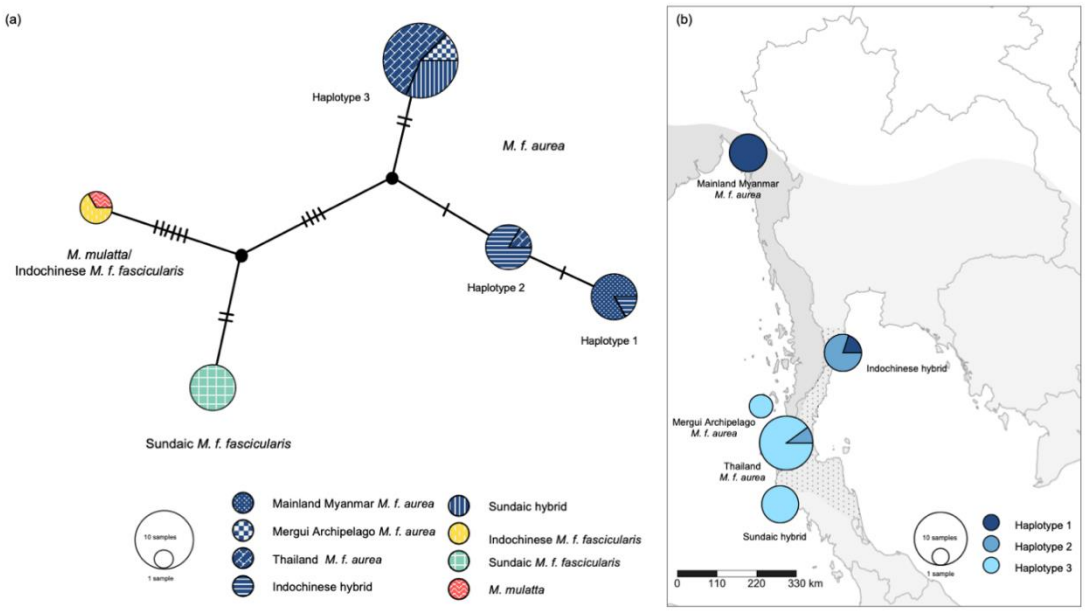


Figure 3.4 (a) The median-joining *TSPY* haplotype network. The short bars on each branches indicate base substitution. (b) Distribution of *M. f. aurea*'s *TSPY* haplotypes in each population of macaques.

Divergence times based on mtDNA sequences

The estimated divergence times based on mtDNA sequences are shown in Figure 3.5 and Table 3.2. The divergence of *M. f. aurea* and *M. mulatta*/*M. f. fascicularis* occurred at around 3.66 MYA (95% HPD CI: 2.19-5.30). Within the *M. f. aurea* clade, mainland Myanmar and Mergui Archipelago/Thailand *M. f. aurea* diverged around 0.99 MYA (95% HPD CI: 0.45-1.58) followed by the diversification of the Mergui Archipelago and Thailand *M. f. aurea* at 0.37 (95% HPD CI: 0.18-0.59). *M. mulatta* and *M. f. fascicularis* diverged at 2.11 MYA (95% HPD CI: 1.16-3.38). Sundaic Thai Gulf *M. f. fascicularis* diverged from Indochinese/Sundaic Andaman Sea Coast *M. f. fascicularis* at 1.39 MYA (95% HPD CI: 0.73-2.09) followed by the divergence of Indochinese and Sundaic Andaman Sea Coast *M. f. fascicularis* at 0.76 MYA (95% HPD CI: 0.39-1.16) and the diversification among Sundaic Thai Gulf *M. f. fascicularis* populations at 0.76 MYA (95% HPD CI: 0.39-1.18).

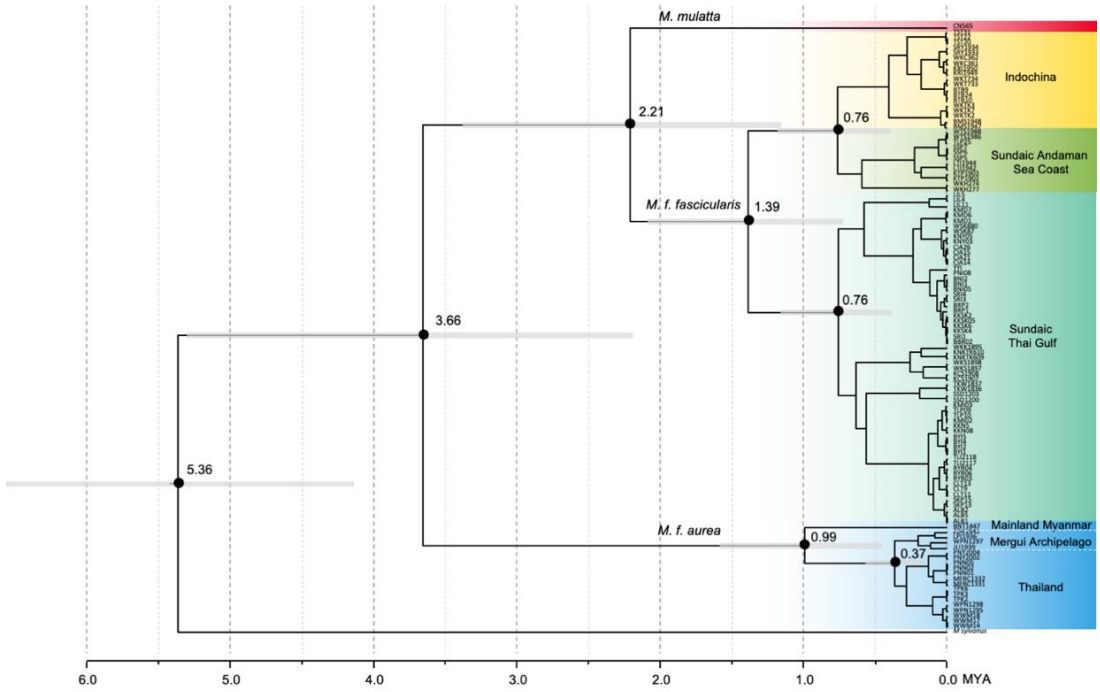


Figure 3.5 The divergence times based on mtDNA data. The values at nodes are mean divergence times (MYA) between each clade. Gray bars at the nodes indicated a 95% HPD credibility interval.

Table 3.2 The divergence times are estimated based on mtDNA data (in MYA).

Node of Interest	Mean	95% HPD CI[b]
M. sylvanus – Asian macaques[a]	5.36	4.13-6.56
M. f. aurea – *M. mulatta*/*M. f. fascicularis*	3.66	2.19-5.30
M. mulatta – *M. f. fascicularis*	2.21	1.16-3.38
Sundaic Thai Gulf *M. f. fascicularis* – Indochinese/Sundaic Andaman Sea Coast	1.39	0.73-2.09
Indochinese – Sundaic Andaman Sea Coast *M. f. fascicularis*	0.76	0.39-1.16
MRCA[c] of Sundaic Thai Gulf *M. f. fascicularis*	0.76	0.39-1.18
Mainland Myanmar – Mergui Archipelago/ Thailand *M. f. aurea*	0.99	0.45-1.58
MRCA of Mergui Archipelago/ Thailand *M. f. aurea*	0.37	0.18-0.59

[a] calibration node.
[b] 95% highest posterior density credibility interval.
[c] Most recent common ancestor

Discussion

M. f. aurea was proposed to be originated from the ancient hybridization between proto-*M. f. aurea* males and the *sinica* species group in modern-day Myanmar or Bangladesh (Matsudaira et al., 2018). Previously, the estimated divergence time based on 2871 bp of Y-chromosome genes indicated that *M. f. aurea* and Sundaic Thai Gulf *M. f. fasicularis* diverged at around 2.58 MYA (95% HPD CI: 1.07–4.02), and the node time of MRCA of *M. f. aurea* estimated based on the whole mitochondrial genome sequence was 0.95 MYA (95% HPD CI: 0.74–1.15, Matsudaira et al., 2018), which was the same range of time estimated based on 674 bp of mtDNA sequences analyzed in this study (0.99 MYA, 95% HPD CI: 0.45-1.58, Figure 3.5, Table 3.2). Taken the results of this study together with that of Matsudaira et al. (2018), *M. f. aurea* is proposed to originate during 2.58 to 0.95 MYA. This male-mediated ancient hybridization had led to the divergence of *M. f. aurea* from *M. f. fascicularis*/*M. mulatta* (Figure 3.2, Bunlungsup et al., 2016) and grouped with the *sinica* species group in mtDNA phylogenetic analysis (Matsudaira et al., 2018; Osada et al., 2021), while the Y-chromosome of *M. f. aurea* was clustered with *M. f. fascicularis* in *fascicularis* species group (Matsudaira et al., 2018). The autosomal genetic analyses emphasized the genetic difference of *M. f. aurea* from the conspecific *M. f. fascicularis* and being more closely related to *M. assamensis*, belonging to the *sinica* species group, than the *fascicularis*/*mulatta* species group (Osada et al., 2021).

The first divergence of mainland Myanmar *M. f. aurea* from Mergui Archipelago/Thailand *M. f. aurea* clade of the mtDNA phylogenetic tree (Figure 3.2) supports the origin of *M. f. aurea* subspecies that should be around Myanmar before migrating to Mergui Archipelago and southwestern Thailand (Fooden 1995; San &

Hamada, 2011; Bunlungsup et al., 2016), which potentially occurred during 0.99 to 0.37 MYA before the diversification of Mergui Archipelago and Thailand *M. f. aurea* populations (Figure 3.5, Table 3.2). This time range falls into the period of EMPT ranging from 1.4 – 0.4 MYA (Head & Gibbard, 2015), or 1.25 – 0.70 MYA in a narrower window (Clark et al., 2006; Chalk et al., 2017). The increase in glacial-interglacial cycle interval and the high amplitude of climate oscillations during EMPT led to the increase in global ice volume in Northern hemisphere (Willeit et al., 2019), strengthened the aridity across the globe including central and northern Asia, and contributed the effect to terrestrial biota composition (Head & Gibbard, 2015) and genetic consequences of animal species (Hewitt, 2004; Othman et al., 2020; Ghane-Ameleh et al., 2021). The impact of cooling climates and environmental changes during the EMPT has been reported in the lineage split and dispersion of Asian black-spined toads (*Duttaphrynus melanostictus*) in Southeast Asia (Othman et al., 2020). Aligned with the origin of *M. f. aurea*, the Southeast Asian lineages of this toad species were hypothesized to originate in Myanmar. Then during the EMPT the coastal lineage diverged from the mainland lineage (1.43-0.84 MYA) and colonized along with coastal Myanmar, southern Thailand, and Peninsula Malaysia at around 0.53 MYA (Othman et al., 2020). Although the EMPT probably affected the divergence of amphibians and mammals differently, this scenario is an empirical example that might also occur in *M. f. aurea* lineages.

In Thailand, six populations of *M. f. aurea*, based on mtDNA and Y-chromosome gene analyses in this study, have been found in only Ranong province ranging from 9°35'N - 10°19'N. This small distribution of Thai *M. f. aurea* provides crucial resources to stone tool use studies in Old World monkeys (Gumert et al., 2009;

Gumert & Malaivijitnond, 2012; Gumert & Malaivijitnond, 2013; Haslam et al., 2016; Falotico et al., 2017; Willeit et al., 2019), and a serious conservative measure should be set up. An intensive sample collection from the intersubspecific hybridization zone in southern Thailand in this study emphasized the existence of the Sundaic Thai Gulf subclade of *M. f. fascicularis* that diverged from Indochina/Sundaic Andaman Sea Coast subclade (Figure 3.2; Bunlungsup et al., 2017a). This subclade was estimated to diverge from Indochina/Sundaic Andaman Sea Coast subclade at around 1.39 MYA (95% HPD CI: 0.73-2.09), which was a bit earlier than those reported previously (1.07 MYA, 95% HPD CI: 0.50–1.76, Bunlungsup et al., 2017a). Later, the diversification among Sundaic Thai Gulf populations occurred at 0.76 MYA (95% HPD CI: 0.39-1.18), which was the same divergent time between Indochinese and Sundaic Andaman Sea Coast subclades in this study (0.76 MYA, 95% HPD CI: 0.39-1.16) and those in Bunlungsup et al. (2017a).

Intersubspecific hybrid populations between *M. f. aurea* and *M. f. fascicularis* in this study were found ranging from 7°50'N - 12°30'N in the peninsula part of Thailand. Bunlungsup et al. (2016) divided hybrid populations into two groups based on their localities and mtDNA haplotypes: Indochinese and Sundaic hybrid groups. Indochinese hybrid populations located north of the Isthmus of Kra carried the Indochinese mtDNA haplotypes of *M. f. fascicularis*, while Sundaic hybrid populations located south of the Isthmus of Kra carried the Sundaic *M. f. fascicularis* mtDNA haplotypes. These two hybrid groups were proposed to be the consequences of two different migration routes of *M. f. aurea* males to the *M. f. fascicularis* populations (Bunlungsup et al., 2016). *M. f. aurea* males migrated from mainland

Myanmar southwardly to the Mergui Archipelago and southern Thailand. Then, some *M. f. aurea* males migrated further south and hybridized with Sundaic *M. f. fasicularis* females in *M. f. fasicularis* populations. Other *M. f. aurea* males migrated northern-eastwardly and hybridized with Indochinese *M. f. fascicularis*. The *TSPY* haplotype network analyzed in this study (Figure 3.4) confirmed this hypothesis. Besides, the results of the *TSPY* gene of *M. f. aurea* discovered in three Sundaic hybrid CIA, BRP, and SRI populations and in two Thailand *M. f. aurea* TPK and WWM populations in this study could strongly support the two different migration routes of male *M. f. aurea* in the hybridization scenarios proposed previously (Bunlungsup et al., 2016).

The intersubspecific *M. f. aurea* x *M. f. fascicularis* hybridization that occurred via male migration of *M. f. aurea* to *M. f. fascicularis* populations was confirmed in this study. Only the mtDNA haplotypes of *M. f. fascicularis* and either the Y-chromosome haplotypes of *M. mulatta*/*M. f. fascicularis* or the *M. f. aurea* in the hybrid populations, but not vice versa, were detected. The coexistence of both Y-chromosome haplotypes, *M. malatta/M. f. fascicularis* and *M. f. aurea*, in Indochinese hybrid populations, was previously reported in Bunlungsup et al. (2016), and this study has confirmed that similar event in Sundaic hybrid populations, i.e., CIA, BBR, KKSK, and SRI, where both *SRY* types of *M. f. fascicularis* and *M. f. aurea* were found in the populations (Figure 3.3). The coexistence of two types of Y-chromosome genes has been explained with two hypotheses (Bunlungsup et al., 2016). The hybridization events that occurred in the recent time or the Y-chromosome haplotypes of *M. f. fascicularis* and *M. f. aurea* have not been selected over each other, although the hybridization occurred a long time ago.

Since this study can further identify the *M. f. aurea* x *M. f. fascicualaris* hybrid populations, either based on morphological characters and mtDNA and *SRY* gene analyses, it is interesting to know if these hybrid macaques can use stone tools to forage for foods, after these behaviors were reported only in two *M. f. aurea* x *M. f. fascicualaris* hybrid populations in Koram Island, Khao Sam Roi Yot National Park (Tan et al., 2017) and Pang-nga Bay National Park (Luncz et al.,2019). The divergence time estimation in this study proposed the potential influence of EMPT on the divergence of *M. f. aurea* lineages. The contradiction of phenotypic and genotypic subspecies of *M. fascicualaris* populations (Table 3.1) suggested that more sensitive genetic markers, e.g., autosomal SNPs, should be used in the future studies.

CHAPTER IV
INFERENCES OF *Macaca fascicularis aurea* POPULATION STRUCTURE AND PATTERNS OF INTRA- AND INTER-SPECIFIC ADMIXTURE USING RADSEQ-DERIVED AUTOSOMAL SNPS

Introduction

As mentioned in Chapter III that *M. f. aurea* originated from the ancient hybridization between proto-*M. f. aurea* males and *sinica* species group females in Myanmar/Bangladesh, leading to the possession of *M. f. aurea* Y-chromosome haplotypes and *sinica* mtDNA haplotypes (Matsudaira et al., 2018). Later, *M. f. aurea* migrated southeastwardly along the Andaman Sea Coast to southwestern Thailand and hybridized with *M. f. fasicularis* females in the Thai peninsula, ranging from 8°10'-12°24'N (Fooden, 1995; Bunlungsup et al., 2016). Genetic studies based on mtDNA and Y-chromosome (*SRY* and *TSPY*) genes in Chapter III also indicated how the intraspecific hybridization occurred (Bunlungsup et al., 2016; Matsudaira et al., 2018).

Apart from the intraspecific hybridization between *M. f. aurea* and *M. f. fascicularis*, the interspecific hybridization between *M. fascicularis* and *M. mulatta* has been reported at 15-20°N across Myanmar, Thailand, Laos, and Vietnam (Fooden, 1964; Hamada et al., 2006; Kanthaswamy et al., 2008; Bonhomme et al., 2009; Stevison & Kohn, 2009; Yan et al., 2011; Bunlungsup, 2017b). Around the Isthmus of Kra, a zoogeographical barrier between the Indochina and Sundaic regions (10°15′N), *M. f. fascicularis* are subdivided into two biogeographical forms based on their genetic differences: the Indochinese cluster carrying the Y-chromosome of *M. mulatta*

and Sundaic cluster carrying the Y-chromosome of *M. f. fascicularis* (Tosi et al., 2002; Bunlungsup et al., 2017a; Rovie-Ryan et al., 2021). Additionally, based on autosomal SNP data, the Indochinese and Sundaic *M. f. fascicularis* exhibit varying levels of *M. mulatta* genetic ancestry, which gradually decline from the north to the south of their distribution range (Bunlungsup et al., 2017b; Ito et al., 2020).

Beyond this information on intraspecific hybridization between *M. f. aurea* and *M. f. fascicularis* and direction of introgression reported in Chapter III, specific knowledge about the events of genetic admixture between *M. f. aurea* and *M. f. fascicularis* is lacking compared to what is known to occur between populations of *M. mulatta* and *M. fascicularis* (Fooden, 1964; Tosi et al., 2002; Hamada et al., 2006; Kanthaswamy et al., 2008; Bonhomme et al., 2009; Stevison & Kohn, 2009; Kanthaswamy et al., 2010; Yan et al., 2011; Kanthaswamy et al., 2013; Satkoski et al., 2013; Bunlungsup, 2017b; Ito et al., 2020; Osada et al., 2021). To gain more information on the genetic structure and composition of *M. f. aurea* and those of *M. f. fascicularis* and *M. mulatta*, a panel of highly informative autosomal SNP markers, was used in this chapter IV.

This study presents the first RADseq-derived autosomal SNP-based assessment of the genetic structure and composition of *M. f. aurea* populations covering their entire distribution range. The levels of genetic admixture among *M. f. aurea*, *M. f. fascicularis*, and *M. mulatta* throughout Thailand, where the core hybridization between the two species and the two subspecies occurred. The RADseq technique works by first fragmenting the target genome using a restriction enzyme. Then, a series of molecular processing steps transform the digested DNA into a fragment library suitable for sequencing on an NGS platform. The RADseq technique is a cost-

effective method to discover SNPs for population and quantitative genetic and phylogeographic studies involving a large number of animals, particularly because genomic resources remain underdeveloped for *M. f. aurea* (Davey & Blaxter, 2010; McCormack et al., 2013; Andrews et al., 2016; Guppy et al., 2020).

Methods

Ethical note

The permit for research and sample collection in Thailand was approved by the NRCT and the DNP. The IACUC of NPRCT-CU approved the study's experimental protocols (Protocol Review no. 1975007). The research adhered to the ASP Principles for the Ethical Treatment of Non-Human Primates.

Sample collection and DNA extraction

In order to accurately illustrate the population structure of *M. f. aurea* and the levels of genetic admixture between species (*M. mulatta* and *M. fascicularis*), between subspecies (*M. f. fascicularis* and *M. f. aurea*), and between the two biogeographical forms of the same subspecies (Indochinese *M. f. fascicularis* and Sundaic *M. f. fascicularis*), nearly all study samples (except for the Chinese *M. mulatta* samples, which were derived from captive *M. mulatta* at the Primate Research Institute (PRI), Kyoto University, Japan) were collected from wild animals with known origins (by GPS coordinates of their habitats).

The wild animals were captured using the trapping and sampling techniques described in Malaivijitnond et al. (2008). The species and subspecies to which these animals belonged were identified based on their morphological characteristics; pelage

color, tail length, cheek hair pattern, and geography (Fooden, 1995, 2000; San et al., 2011; Bunlungsup et al., 2016). *M. mulatta* individuals exhibited the bipartite pattern of the pelage color and RLT of less than 70% (Fooden, 2000; Hamada et al., 2016). By contrast, *M. fascicularis* individuals did not display a bipartite pattern of the pelage color, and their RTL was greater than 90% (Fooden, 1964; 1995; Hamada et al., 2016). As mentioned earlier, *M. f. fascicularis* individuals were distinguished from *M. f. aurea* individuals based on transzygomatic or infrazygomatic facial crest hair patterns. Hybrid individuals showed the mixed morphological characteristics between species or subspecies mentioned above.

The study involved a total of 380 individuals from 15 macaque populations (Table 4.1; Figure 4.1), including two *M. mulatta* populations (PRI and BSS), nine *M. f. fascicularis* populations of Indochinese (WHM, TST, WTM, WKT, SSD) and Sundaic (WSK, WKK, KNKTK, and WKH) forms (Fooden 1995; Bunlungsup et al. 2016), and four *M. f. aurea* populations (BNT, WWM, WPN, and MFRC). Fresh whole blood samples were collected from WHM, TST, WSK, WKK WWM, and MFRC for this study. Genomic DNA (gDNA) was extracted from the buffy coat using the Gentra Puregene Blood kit from Qiagen (Hilden, Germany) after the buffy coat, plasma, and red cells were separated by centrifugation at 1000 xg for 10 min. The remaining gDNA samples were retrieved from the DNA archive at the National Primate Research Center of Thailand, Chulalongkorn University (NPRCT-CU), Saraburi, Thailand. These gDNA samples had been purified using either the Gentra Puregene Blood kit or the standard phenol-chloroform method (Sambrook et al., 1989; Malaivijitnond et al., 2008; Bunlungsup et al., 2016; Bunlungsup, 2017a, b).

Table 4.1 Sample size (N) and GPS coordinates of samples used in this study. Presented are observed (H_O) and expected (H_E) heterozygosity, inbreeding coefficient (F_{IS}), and proportion of admixture based on 868 SNPs among three taxa. Note that the populations that do not have a stated country name are from Thailand. *Mm*: *Macaca mulatta*, *Mff*: *M. fascicularis fascicularis*, and *Mfa: M. f. aurea*. † samples collected in this study.

Taxon	**Location**	**Code**	**N**	**GPS (N, E)**	**H_O**	**H_E**	**F_{IS}**	**Average percentage of admixture**		
								Mm	***Mff***	***Mfa***
Chinese *Mm*	Primate Research Institute, Kyoto University, Japan	PRI	30	-	0.268	0.206	-0.321	100	0	0
Thai *Mm*	Ban Sang School	BSS	30	17°51', 103°57'	0.284	0.230	-0.265	100	0	0
Indochinese *Mff*	Wat Had Moon	WHM†	29	16°51', 100°28'	0.281	0.225	-0.273	97	3	0
	Ta Sang Tai	TST†	32	15°56', 99°57'	0.199	0.179	-0.137	81	8	11
	Wat Thammasala	WTM	27	13°48', 100°06'	0.183	0.149	-0.289	95	4	1
	Wat Khao Thamon	WKT	30	13°02', 99°57'	0.230	0.200	-0.181	83	4	14
Sundaic *Mff*	Suan Somdetch Prasrinakarin	SSD	13	9°56', 99°02'	0.283	0.260	-0.127	19	69	12
	Wat Suwan Khuha	WSK†	30	8°25', 98°28'	0.266	0.203	-0.314	1	92	7
	Wat Khao Kaew Wichian	WKK†	26	8°12', 100°05'	0.349	0.261	-0.350	2	98	0
	Khao Noi/Khao Tangkuan	KNKTK	31	7°12', 100°35'	0.248	0.206	-0.227	1	99	0
	Wat Kuha Pimuk	WKH	16	6°31′, 101°13′	0.360	0.274	-0.338	1	99	0
Mfa	Bayin Nyei Temple, Myanmar	BNT	13	16°58', 97°29'	0.444	0.321	-0.411	2	0	98
	World War Museum	WWM†	31	10°10', 98°43'	0.335	0.247	-0.367	1	0	99
	Wat Paknam Pracharangsarit	WPN	6	9°57', 98°35'	0.524	0.400	-0.366	1	2	97
	Mangrove Forest Research Center	MFRC†	35	9°52', 98°36'	0.293	0.215	-0.370	1	2	97
Mean estimates across		*Mm* populations			0.276	0.218	-0.293	100	0	0
		Mff populations			0.267	0.217	-0.248	42	53	6
		Mfa populations			0.400	0.300	-0.380	1	1	98
		All populations			0.167	0.134	-0.289	39	32	29

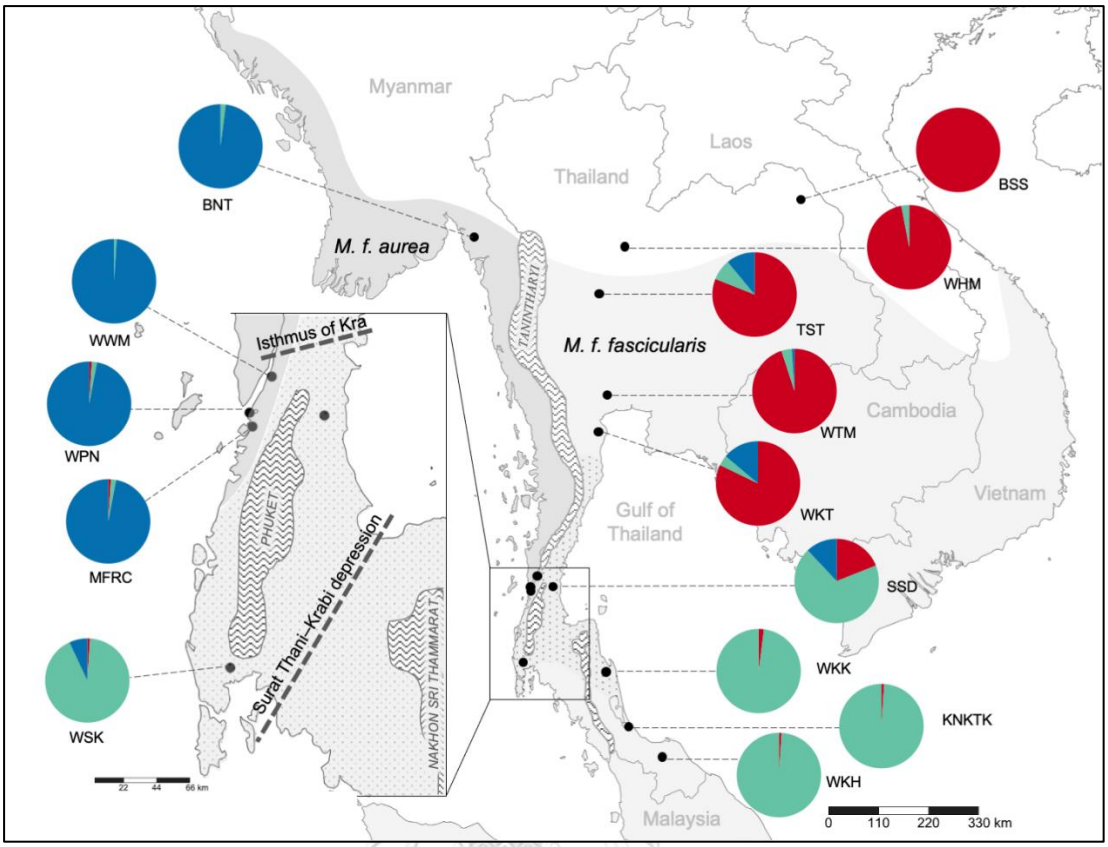


Figure 4.1 Distribution range of *M. f. aurea* (dark gray), *M. f. fascicularis* (gray), and hybrid (dot) based on Fooden (1995) and Bunlungsup et al. (2016). Letters are the population codes that correspond to Table 4.1, except that the PRI *M. mulatta* population is not presented on the map. Pie charts represent the proportion of genetic ancestry in each population; colors correspond to those in Figure 4.2a (K=3).

Sequencing and SNP selection

The gDNA samples from *M. f. aurea*, *M. f. fascicularis*, and *M. mulatta* were submitted to Floragenex Inc. (Oregon, USA) for RADseq library preparation and sequencing. The transfer of the gDNA samples from Thailand to the United States was authorized through a Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) permit (no. 19TH0902.2) issued by the DNP.

For RADseq analysis, the gDNA was digested using the *Sbfl* restriction enzyme to create 300-500 base pairs (bp) fragments for library preparation. The fragments were sequenced on an Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA) to obtain 1x100 bp reads. The Stacks 2.5 software pipeline (Rochette and Catchen, 2017) was used to filter and call SNPs by aligning to the *M. fascicularis* reference genome (Macaca_fascicularis_5.0, GCA_000364345.1) under the following stringent settings: 90% minimum genotyping rate, a minimum PHRED quality score of 20 for individual genotypes, and a minimum sequencing depth of 14x per individual.

Genetic structure and admixture analyses

Arlequin v3.5.1.3 (Excoffier et al., 2005) was used to test for linkage disequilibrium (LD) and Hardy Weinberg Equilibrium (HWE) based on all SNP markers that met the stringent genotyping quality criteria described above. The Arlequin program was used to estimate SNP allele frequency (where p_i is the allele frequency for i^{th} allele of a locus), observed heterozygosity (H_O), expected heterozygosity (H_E), pairwise genetic differentiation (pairwise F_{ST} or pF_{ST}), and inbreeding (F_{IS}) across all 15 populations based on samples with at least 90%

complete data. The same program was used to perform an AMOVA test for each population.

STRUCTURE 2.3.4 (Pritchard et al., 2000) was used to estimate the proportion of genetic admixture *M. f. aurea*, *M. f. fascicularis*, and *M. mulatta* populations. STRUCTURE facilitates the selection of the most likely number of independent taxonomic units represented by the data, and it estimates each animal's fractional membership in each taxon. Proportions of admixture were calculated for each study sample by assigning proportionate ancestry to those samples from the three taxa included in this study. Using the admixture model, simulations were performed with 500,000 iterations after a burn-in period of 100,000 steps. The simulations were performed with and without *a priori* location information (LOCPRIOR) to ensure that the pre-defined populations were in agreement with the genetic data (Hubisz et al., 2009).

Although three macaque taxa were included in this study, the number of genetic clusters could potentially be four (i.e., *M. mulatta*, Indochinese *M. f. fascicularis*, Sundaic *M. f. fascicularis*, and *M. f. aurea)*. This analysis, therefore, was performed with the number of genetic groups (K) ranging from one to seven. In other words, three or more populations were included to account for potential ancestors in other groups (Day et al., 2018). Each K value was run with five replicates, and the results were combined and visualized using CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007) and Distruct 1.1 (Rosenberg, 2007), respectively. The STRUCTURE likelihood function [L(K)] is correlated with the strength of the genetic subdivision among the study populations (Evanno et al., 2005). Therefore, a deltaK (ΔK) analysis based on the second-order derivative of the change in variance of the log probability between

successive K values was used to estimate the number of genetic groups (K) presented in this study. The K value with the highest Ln P(D) and the lowest standard deviation coincides with the highest ΔK (Evanno et al., 2005). To obtain a clear pattern of genetic relationship among the populations sampled in this study, a maximum likelihood (ML) phylogenetic analysis was performed using the GTGTR+I+G model implemented in the RAxML-NG program (Kozlov et al., 2019).

As the effects of gene flow (admixture) cannot be easily distinguished from those of shared ancestral variation since both scenarios can result in a similar pattern of allele sharing, the rates of gene flow were analyzed in a biogeographical setting by comparing the pF_{ST} values of neighboring pairs of populations with those from more distantly located population pairs regardless of which taxon the animals belong. If gene flow is the predominant evolutionary force that shaped the population structure of the different taxa studied here, then differentiation of neighboring population pairs should be lower than that of distantly located population pairs.

Results

The natural habitats in Thailand from which the *M. fascicularis* samples were collected encompass (i) the proposed hybrid zones between *M. f. aure*a and *M. f. fascicularis* and between *M. fascicularis* and *M. mulatta* (Fooden, 1995; 2000; Bunlungsup et al., 2016; Hamada et al., 2016, Table 4.1; Figure 4.1), and (ii) the separation between the two genetically distinct forms of Indochinese and Sundaic *M. f. fascicularis* (Fooden, 1995; Bunlungsup et al., 2017a). A total of 768,528 candidate SNPs were discovered using the RADseq method. Only 1,351 passed the quality filters described in the methods section. After removing all SNPs that did not map to

an autosome in the *Macaca_fascicularis*_5.0 reference genome and the 11 animals (four WKT individuals, two SSD individuals, one BNT individual, and four SSD individuals) with less than 90% of marker data, only 868 of the quality-filtered SNPs were suitable for population genetic analysis (see https://docs.google.com/a/asu.edu/viewer?a=v&pid=sites&srcid=YXN1LmVkdXxrYW50aGFzd2FteS1kbmEtbGFifGd4OjdiYzYzODA3NGVhNWZhYTk). Allele frequencies (p_i) for each SNP in each population are shown in the online platform (see https://docs.google.com/viewer?a=v&pid=sites&srcid=YXN1LmVkdXxrYW50aGFzd2FteS1kbmEtbGFifGd4OjdjOTk2MWQyYWRmNTU3YWU). Estimates of H_E, H_O, and F_{IS} based on the 868 SNP markers are provided in Table 4.1. Interestingly, the *M. f. aurea* populations exhibited much higher averaged levels of genetic diversity (H_O = 0.400 and H_E = 0.300) and lower levels of inbreeding (F_{IS} ranged from -0.366 to -0.411) compared to the *M. f. fascicularis* populations (H_O = 0.267, H_E = 0.217, and F_{IS} estimates ranged from -0.127 to -0.355). The *M. mulatta* populations (H_O = 0.276, H_E = 0.218, and average F_{IS} value = -0.293) displayed genetic diversity metrics intermediate between the *M. f. aurea* and *M. f. fascicularis* populations. All three taxa's negative F_{IS} metrics indicated that none were characterized by prevalent inbreeding. Among the *M. f. aurea* populations, the least genetically diverse population was MFRC, which exhibited H_O and H_E estimates of 0.293 and 0.215, respectively. In contrast, WPN showed the highest H_O and H_E estimates of 0.524 and 0.400, respectively. Among *M. f. fascicularis* populations, WKH showed the highest H_O and H_E estimates of 0.360 and 0.274, while WTM showed the lowest values at H_O = 0.183 and H_E = 0.149, respectively. Despite their relative isolation from *M. mulatta* and *M. f. aurea*, the

Sundaic WSK, WKK, KNKTK, and WKH *M. f. fascicularis* populations possessed much higher levels of genetic diversity than other *M. f. fascicularis* populations.

AMOVA of the 369 individuals permitted a partitioning of overall genetic variation into three levels: among taxa, among populations within each taxon, and across individuals within each population. Based on this analysis, genetic variation among individuals within each population ranged from 92% (*M. f. fascicularis*) to 95% (*M. f. aurea* and *M. mulatta*). In comparison, the proportion of variation among populations within each taxon was approximately 5% or less. The average pF_{ST} estimates revealed slightly higher genetic differentiation between *M. f. fascicularis* and *M. f. aurea* populations (pF_{ST} = 0.074) than between *M. f. fascicularis* and *M. mulatta* populations (pF_{ST} = 0.070) (Table 4.2). However, the *M. mulatta* and *M. f. aurea* divergence was almost twice (pF_{ST} = 0.131) that of *M. f. fascicularis*-*M. f. aurea* and *M. f. fascicularis*-*M. mulatta*.

Pairwise genetic differentiation among populations was most pronounced between the BNT *M. f. aurea* population and all other non-*M. f. aurea* populations, with the highest pF_{ST} value found between BNT and WKH *M. f. fascicularis* (pF_{ST} = 0.191; Table 4.2). The lowest pF_{ST} values were detected among *M. f. aurea* populations, especially between Thai WPN and MFRC *M. f. aurea* populations (pF_{ST} = 0.017). The average pF_{ST} value across all population pairs was only 0.106. The ΔK analysis on the STRUCTURE simulations with K set between one to seven showed a maximum likelihood when K=3, whether sample location information was considered or not (Figure 4. 2). The K=3 STRUCTURE plot running with *a priori* location information is shown in Figure 4.2. The K=4 STRUCTURE plot, if four groups of animals (*M. mulatta*, Indochinese *M. f. fascicularis*, Sundaic *M. f. fascicularis*, and *M.*

f. aurea) were considered, was also presented for comparison. The determination that three is the most likely number of genetic clusters under both settings, i.e., K=3 or K= 4, is consistent with an *M. mulatta*-Indochinese *M. f. fascicularis*, Sundaic *M. f. fascicularis,* and *M. f. aurea* trichotomy (Figure 4.3).

The average ranges of assignment probabilities across geographic samples to their alleged ancestries are shown in Table 4.1. The *M. f. aurea* populations were largely genetically isolated from the *M. f. fascicularis* and *M. mulatta* populations, with individuals being assigned to the *M. f. aurea* cluster with probabilities ranging from 97 to 99% (population-specific averages were between 97% and 99%). Based on the STRUCTURE analysis, when K=3, the *M. f. fascicularis* populations were subdivided into two genetic clusters of Indochina and Sunda, where the Indochinese WHM, TST, WTM, and WKT *M. f. fascicularis* populations affined more strongly (81-97%) to the PRI and BSS *M. mulatta* populations (Table 4.1 and Figure 4.2a).

In agreement with the STRUCTURE analysis, the ML analysis showed the three major clades of *M. mulatta*/Indochinese *M. f. fascicularis*, Sundaic *M. f. fascicularis*, and *M. f. aurea* (Figure 4.3). While the formation of the *M. mulatta*/Indochinese *M. f. fascicularis* clade illustrated the existence of gene flow between *M. mulatta* and the Indochinese *M. f. fascicularis,* individuals belonging to each of these species within this clade fell into two separate subclades. The *M. mulatta*-Indochinese *M. f. fascicularis* separation was also reproduced when the STRUCTURE analysis was rerun under the assumption that K=4 (Figure 4.2a), stressing the genetic distinction between these two species of macaques despite ongoing admixture (Kanthaswamy et al. 2008; Bunlungsup 2017a, b).

Allele sharing between the captive Chinese PRI *M. mulatta* and the wild-caught Thai (BSS) *M. mulatta* is unlikely to have arisen independently. Therefore, the shared ancestral variation cannot be ruled out as having caused some of the reduced levels of genetic differentiation observed among populations. Furthermore, there was no significant difference between neighboring population pairs and more distant population pairs ($p = 0.57$; Mann–Whitney U-test), suggesting that genetic introgression alone did not influence the genetic structure and population stratification observed here. Thus, low genetic differentiation, where most alleles are in common between populations, could have resulted from the competing effects of shared ancestry and low admixture rates.

Table 4.2 Pairwise F_{ST} values based on 868 SNPs between populations of *Macaca mulatta (Mm)*, *M. fascicularis fascicularis (Mff)*, and *M. f. aurea* (*Mfa*). All pF_{ST} values are significant at the p = 0.05 level.

		Mm	**Indochinese *Mff***					**Sundaic *Mff***					***Mfa***		
		PRI	BSS	WHM	TST	WTM	WKT	SSD	WSK	WKK	KNKTK	WKH	BNT	WWM	WPN
Mm	BSS	0.053													
Indochinese *Mff*	WHM	0.101	0.093												
	TST	0.103	0.081	0.060											
	WTM	0.106	0.092	0.083	0.051										
	WKT	0.110	0.095	0.077	0.047	0.058									
Sundaic *Mff*	SSD	0.117	0.097	0.088	0.039	0.058	0.042								
	WSK	0.130	0.115	0.096	0.072	0.083	0.088	0.049							
	WKK	0.139	0.124	0.107	0.085	0.104	0.101	0.066	0.063						
	KNKTK	0.142	0.122	0.118	0.078	0.094	0.099	0.050	0.052	0.071					
	WKH	0.159	0.146	0.145	0.105	0.119	0.122	0.077	0.067	0.088	0.062				
Mfa	BNT	0.184	0.188	0.160	0.133	0.164	0.152	0.143	0.148	0.158	0.180	0.191			
	WWM	0.167	0.160	0.126	0.102	0.127	0.117	0.100	0.109	0.120	0.143	0.161	0.079		
	WPN	0.166	0.164	0.146	0.109	0.135	0.128	0.094	0.098	0.121	0.131	0.139	0.069	0.033	
	MFRC	0.146	0.137	0.111	0.086	0.108	0.094	0.081	0.092	0.098	0.120	0.131	0.075	0.030	0.017

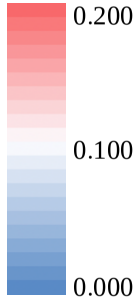


Figure 4.2. Genetic structure analysis based on 868 SNPs. (a) The K=3 STRUCTURE plot run with *a priori* location information shows the proportion of *M. mulatta*-Indochinese *M. f. fascicularis* (red), Sundaic *M. f. fascicularis* (green), and *M. f. aurea* (blue) ancestry in each population. Also shown is the K=4 STRUCTURE plot, which can distinguish *M. mulatta* (red) and Indochinese *M. f. fascicularis* (yellow). (b) ΔK analysis for values of K in the STRUCTURE analysis reveals that K=3.

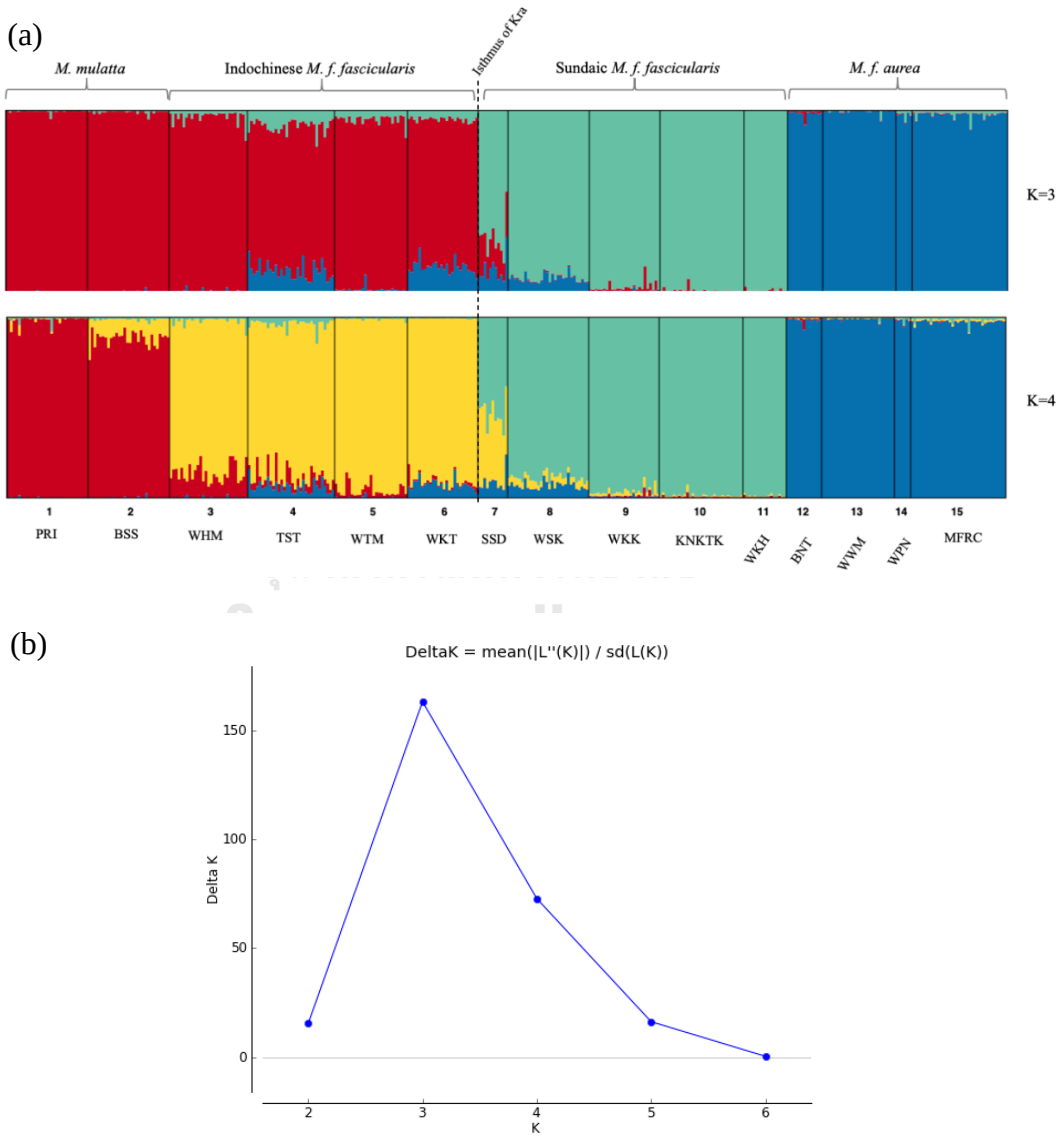
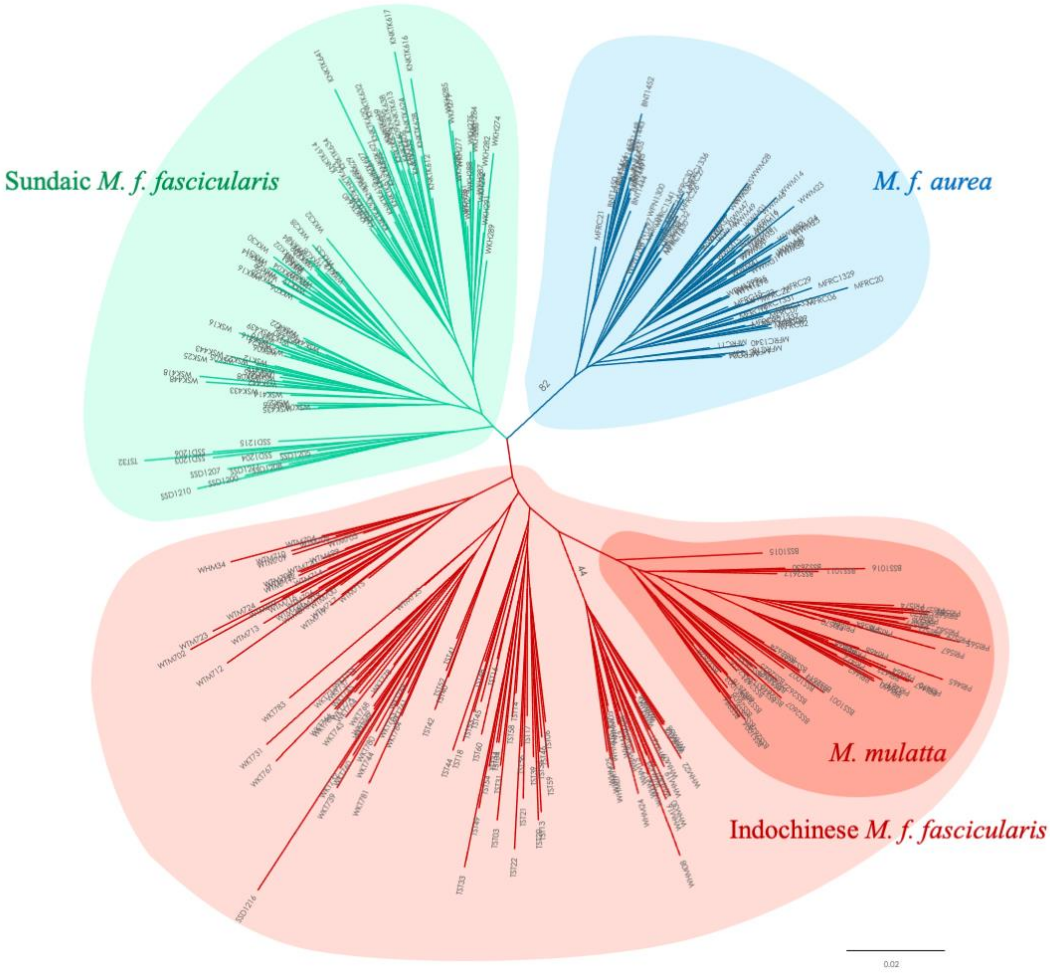


Figure 4.3 The ML phylogenetic tree of 369 individuals based on 868 autosomal SNP loci was included in the study. The ML tree was constructed using RAxML-NG (Kozlov et al., 2019) with the GTGTR+I+G model. One hundred and fifty bootstraps were performed, and only the values above 40 are shown. The tree indicates three distinct clades: *M. mulatta*/Indochinese *M. f. fascicularis* (red), Sundaic *M. f. fascicularis* (green), and *M. f. aurea* (blue).



Accordingly, genetic introgression from the *M. f. aurea* ancestry was not detected in the PRI and BSS *M. mulatta* populations, nor in the northernmost WHM Indochinese *M. f. fascicularis* population living at 16°51'N. However, *M. f. aurea* ancestry ranging from 1 to 14% was observed in several *M. f. fascicularis* populations (Figure 4.2a). The three Indochinese *M. f. fascicularis* populations, TST (15°56'N), WTM (13°48'N), and WKT (13°02'N), exhibited 11%, 1%, and 14% *M. f. aurea* ancestry, respectively. The low *M. f. aurea* ancestry percentage in WTM was attributed to the presence of a single hybrid individual sampled at that study site (Figure 4.2a). Two Sundaic *M. f. fascicularis* populations, SSD (9°56'N) and WSK (8°25'N), which live in the designated intraspecific hybrid zone (8°10'-12°24'N; Fooden 1995), exhibited *M. f. aurea* ancestry at levels of 12% and 7%, respectively. In contrast, the Sundaic *M. f. fascicularis* populations of WKK (8°12'N), KNKTK (7°12'N), and WKH (6°31′N) located in southeastern Thailand showed almost exclusively *M. f. fascicularis* ancestry with no genetic admixture of *M. f. aurea*. No genetic introgression from *M. f. fascicularis* was detected within the BNT population, which was the only *M. f. aurea* population living in Myanmar that was include in this study. Although introgression from the *M. f. fascicularis* ancestry was detected in two Thai WPN and MFRC *M. f. aurea* populations, the level of admixture was minimal (approximately 2%).

Discussion

Genotypic information from 868 autosomal SNPs was used to examine the genetic structure and composition of *M. f. aurea* and the degree of genetic admixture between *M. mulatta*, *M. f. fascicularis*, and *M. f. aurea*. STRUCTURE analysis

revealed an Ln P(D) and ΔK showing the highest probability at K=3, commensurate with the three putative genetic groups: *M. mulatta*-Indochinese *M. f. fascicularis*, Sundaic *M. f. fascicularis*, and *M. f. aurea*. The ML phylogenetic networks also revealed a similar trichotomy pattern among the different macaque taxa investigated. The strong genetic affinity between *M. mulatta* and Indochinese *M. f. fascicularis* indicated in this study illustrates the influence of *M. mulatta – M. f. fascicularis* interspecific introgression on macaques' genetic make-up in the Indochinese region. Nonetheless, the taxonomically recognized taxa, i.e., *M. mulatta, M. f. aurea*, *M. f. fascicularis*, can clearly be distinguished, and the two forms of *M. f. fascicularis* were also pronounced when K=4 was set. The genetic distinction of *M. f. aurea* in this study is consistent with Matsudaira et al. (2018) and Osada et al. (2021), who concluded that *M. f. aurea* arose through hybridization between a lineage with ancestry close to *M. fascicularis* and a lineage that was part of the *sinica* species group of macaques. In fact, in phylogenetic analyses, *M. f. aurea* carried mtDNA haplotypes that clustered with the *sinica* species group rather than *M. fascicularis* (Matsudaira et al., 2018; Osada et al., 2021).

The overall mean pF_{ST} estimate of 0.106 generated among the different population pairs reflects low genetic differentiation, which could have resulted from shared ancestry and low rates of gene flow. Additionally, the frequency of alleles common to multiple populations may differ due to genetic drift and/or mutations occurring independently in each population which can operate against the homogenizing effect of gene flow and cause random allele frequency shifts (Hartl 1999). Based on the pF_{ST} estimates, samples of *M. f. fascicularis* were more closely affined to *M. mulatta* (0.070) than their conspecific *M. f. aurea* (0.074). When

STRUCTURE assumed a K value of three, individuals from both *M. fascicularis* subspecies exhibited *M. mulatta* ancestry. However, none of the *M. mulatta* subjects showed any trace of *M. fascicularis* ancestry (Table 4.1). This finding supports numerous studies that have proposed a primarily unidirectional introgression from *M. mulatta* to *M. fascicularis* in mainland Southeast Asia, mainly driven by male-mediated gene flow (Tosi et al. 2002; Kanthaswamy et al. 2008; Bonhomme et al. 2009; Stevison and Kohn 2009; Yan et al. 2011; Bunlungsup et al. 2017a). In the present study, genetic introgression from *M. mulatta* into *M. fascicularis* correlated longitudinally with geographic distance far from the proposed interspecies hybridization zone (15-20°N). Using autosomal SNP markers, Bunlungsup et al. (2017b) also reported that the highest level of the genetic introgression from *M. mulatta* to *M. fascicularis* was in the proposed interspecific hybrid zone, and the level of interbreeding between the congeneric taxa gradually declined southwardly from the hybrid zone.

The genetic similarity between Indochinese *M. f. fascicularis* and *M. mulatta* populations was undoubtedly high (Table 4.2; Figure 4.2a) due to the overrepresentation of *M. mulatta* alleles in Indochinese WHM, TST, WTM, and WKT *M. f. fascicularis* populations living at 16°51'-13°02'N compared to their Sundaic SSD, WSK, WKK, KNKTK, and WKH conspecific populations living at 9°56'N to 6°31'N. The ML tree also showed a similar pattern; the WHM, TST, WTM, and WKT Indochinese *M. f. fascicularis* populations were more closely related to *M. mulatta* than any other population. Varying *M. mulatta* introgression rates due to various natural barriers, including topographical features and disease susceptibility, could have augmented the pronounced differentiation between the Indochinese and Sundaic

forms of *M. f. fascicularis* populations (Tosi et al., 2002; Hughes et al., 2011; Bunlungsup et al., 2016; Zhang et al., 2017; Rovie-Ryan et al., 2021).

In addition to the well-known hybridization scenario between *M. mulatta* and *M. fascicularis* at 15-20°N, the proportion of *M. mulatta* admixture into Thai *M. f. aurea* populations (9°52'-10°10'N) indicated plausible male-mediated introgression of *M. mulatta* into *M. f. aurea* populations. However, the admixture values were relatively small (1-2%). Consequently, the differentiation between *M. f aurea* and *M. mulatta* (pF_{ST} = 0.131) was almost double that of *M. f. fascicularis* and *M. mulatta* (pF_{ST} = 0.07). Gene flow from *M. mulatta* was detected up to the BNT *M. f. aurea* population in Myanmar (16°58'N), which is genetically divergent from the Thai *M. f. aurea* populations. This finding may support Bunlungsup et al.'s (2016) and the results in Chapter III claim that members of the BNT population were the carriers of the oldest *M. f. aurea* mtDNA haplotypes, consistent with the hypothesis that *M. f. aurea* originated in Myanmar (San and Hamada, 2011).

This SNP study showed that the hybridization between *M. f. fascicularis* and *M. f. aurea* extended beyond the intraspecific hybrid zone (8°10'-12°24'N) previously proposed by Fooden (1995). Unlike the hybridization between *M. mulatta* and *M. fascicularis,* which occurred only along the north-south direction (Table 4.1, Figure 4.2a), introgression between *M. f. fascicularis* and *M. f. aurea* occurred in two directions: south to north (8°25' to 15°56') and west to east (98°28' to approximately 99°; i.e., 99°02' for the SSD population and 99°57' for the TST population, respectively). However, the direction of gene flow was mainly upward from the south to the north because *M. f. aurea* ancestry in Indochinese *M. f. fascicularis* populations terminated at the TST site (15°56').

Bunlungsup et al. (2016) proposed the hybridization events that *M. f. aurea* males from Myanmar may have taken two possible migration routes: an earlier southwardly one along the Mergui archipelago and the Andaman Sea coast toward southwestern Thailand, where they mated with Sundaic *M. f. fascicularis* females; and a later eastwardly one across low-altitude areas of the Tanintharyi range to the Gulf of Thailand, where they mated with Indochinese *M. f. fascicularis* females (San and Hamada, 2011; Bunlungsup et al., 2016). In the current study, the *M. f. aurea* ancestry in Sundaic WSK *M. f. fascicularis* population (8°25'N; 7%) supports the hybridization event caused by the southward migration of *M. f. aurea* from Myanmar, and the Sundaic SSD *M. f. fascicularis* population (9°56'N; 12%) supports the hybridization event caused by the eastward migration of *M. f. aurea* from the Andaman Sea coast.

These results indicate that the Surat Thani-Krabi depression might be a zoogeographical barrier for the migration of southwestern *M. f. aurea* to the eastern side. Thus, the west-east direction of the hybridization between *M. f. aurea* and *M. f. fascicularis* could not occur across the Surat Thani-Krabi depression. The strong influence of this geographical feature on *M. fascicularis* subdivision was also previously reported to have resulted in the distribution limit between the *M. leonina* (northern pig-tailed macaque) and *M. nemestrina* (southern pig-tailed macaque; Malaivijitnond et al., 2012). The high level of *M. f. aurea* ancestry among the Indochinese WKT *M. f. fascicularis* (13°02'N; 14%) likely supports the more recent hybridization event caused by the eastward *M. f. aurea* migration from Myanmar to the Gulf of Thailand.

Given the presence of a higher proportion of *M. f. aurea* ancestry in *M. f. fascicularis* individuals (up to 14%) than in the opposite direction (up to 2%), this

study depicted an asymmetric introgression pattern between *M. f. aurea* and *M. f. fascicularis* populations. Bunlungsup et al. (2016) proposed that the hybridization events between *M. f. fascicularis* and *M. f. aurea* occurred primarily by *M. f. aurea* male-mediated gene flow into *M. f. fascicularis* populations and that the *M. f. aurea* and *M. mulatta*/*M. f. fascicularis* patrilines have coexisted in Indochinese and Sundaic *M. f. fascicularis* populations. This implies that the *M. f. aurea* gene flow into *M. f. fascicularis* populations was more recent, and/or a lack of selective advantage of *M. f. aurea* males over *M. f. fascicularis* males (Osada et al., 2010; Bunlungsup et al., 2016). The absence of *M. f. aurea* ancestry in the Sundaic WKK, KNKTK, and WKH *M. f. fascicularis* populations on the eastern side of the southern Thai peninsula demonstrates that the Phuket Range (9°20N, 98°37′E), which is a continuation of the greater Tanintharyi Range, and the Nakhon Si Thammarat Range (8°30′N, 99°34′E), which lies in a north-south orientation on the peninsula, might be additional barriers to genetic admixture between *M. f. aurea* and *M. f. fascicularis*.

This large-scale SNP study also underscores the role of other natural factors in the distribution of *M. f. aurea* genetic variation in Thailand. This study is the first to report the possibility of genetic introgression of *M. f. aurea* into Indochinese *M. f. fascicularis* located in central Thailand (TST; 15°56'N, 99°57'E), which is approximately 500 km north of the previously proposed intraspecific hybridization zone (8°10'-12°24'N; Fooden, 1995; Bunlungsup et al., 2016). Although shared ancestral variation cannot be discounted as having played a role in the similarity between the *M. f. aurea* and the TST Indochinese *M. f. fascicularis* population, the fact that the WKT and TST populations contained an average of more than 10% *M. f. aurea* admixture reflects the effects of hybridization between the two *M. fascicularis*

subspecies. Generally, *M. f. aurea* is distributed along coastal regions and mostly inhabited island, mangrove, riverine lowland forest, or coastal hill habitats (Fooden, 1995; San and Hamada, 2011; Bunlungsup et al., 2016). It remains questionable whether the presence of *M. f. aurea* ancestry in the WKT and TST *M. f. fascicularis* population was due to natural introgression or caused by human-assisted animal translocation.

Although the degrees of hybridization and gene flow of *M. mulatta*/*M. f. fascicularis* alleles into *M. f. aurea* were low, this study detected that the genetic diversity indices calculated from *M. f. aurea* were much higher than those of *M. mulatta* and *M. f. fascicularis.* Moreover, the AMOVA indicated that *M. f. aurea* harbored a greater percentage of variation within populations than *M. mulatta* and *M. f. fascicularis*. Concomitantly, *M. f. aurea* also exhibited low inbreeding levels, followed by *M. mulatta* and *M. f. fascicularis*. Besides the issues of the hybridization between *M. mulatta* and *M. f. fascicularis* and the genetic divergence of Indochinese and Sundaic *M. f. fascicularis*, this study reports the genetic admixture between *M. f. aurea* and *M. f. fascicularis* in both the Indochina and Sunda regions and the genetic variation between species, subspecies, forms and populations of *M. mulatta* and *M. fascicularis*.

The results of 868 autosomal SNP-based assessments of the genetic structure and composition of *M. f. aurea* in this study will allow the future development of an SNP assay to facilitate a more efficient genotyping workflow that could effectively distinguish between *M. f. fascicularis* and *M. f. aurea.* Comparable strategies are now achievable that utilize inter-fluidic PCR chips to amplify up to 96 SNP markers at

once and quickly discover population admixture in macaques (Zhang et al., 2017; Day et al., 2018).

CHAPTER V
SUBSPECIES STATUS AND DEGREE OF GENETIC ADMIXTURE OF *Macaca fascicularis aurea* IN ASSOCIATION WITH STONE-ASSOCIATED BEHAVIORS

Introduction

Apart from chimpanzees (*Pan troglodytes*) and capuchins (*Sapajus libidinosus* and *S. xanthrosternos*) (Haslam et al., 2009; Wynn et al., 2011), Burmese long-tailed macaques (*Macaca fascicularis aurea*) are another non-human primate species that habitually use stones as tools to forage for foods (Malaivijitnond et al., 2007; Gumert et al., 2009). The stone-tool use behavior in *M. f. aurea* was rediscovered at Piak Nam Yai island (9°34' N, 98°28' E), Ranong, Thailand (Malaivijitnond et al., 2007) after over 120 years of the first brief report in Mergui Archipelagos, Myanmar (Carpenter, 1887). *M. f. aurea* are the only Old World monkeys among other stone-tool-use non-human primates (Lunzc et al., 2019) that use stones to process various types of encased foods, e.g., mollusks, crustaceans, and nuts (Gumert and Malaivijitnond, 2012; Lunzc et al., 2017). Although *M. f. fascicularis* is a conspecific species that lives closely in southwestern Thailand, no stone tool use behaviors have been reported in wild or captive *M. f. fascicularis* (Malaivijitnond and Hamada 2008; Malaivijitnond et al. 2011; Bandini and Tennie, 2018). Noted, the hybrids between *M. f. aurea* and *M. f. fascicularis* living on Koram island (12°14′N, 100°0'E) and in Ao Phang-nga National Park (8°10′N, 98°37'E), Thailand have been reported using stone tools (Bunlungsup et al., 2016; Tan, 2017). Among the non-stone-tool users, the stone-assisted behaviors, such as food pounding and stone-play, were reported. For food

pounding behavior, monkeys perform a direct percussion of food items onto hard substrates, e.g., unmanipulated embedded stones, as seen in Koram Island macaques, Sam Roi Yot National Park, Prachuap Khiri Khan, Thailand (Tan, 2017). Stone-play behavior is a manipulation of stone in different ways, e.g., rolling and rubbing (Huffman, 1984; Nahallage & Huffman, 2008) to the substrates as seen in Wat Khao Takieb macaques (12°30'N, 99°58'E), Prachuap Khiri Khan, Thailand (Carter et al., 2016). Thus, stone-play behavior is considered a potential behavioral precursor of stone-tool use behaviors (Huffman & Quiratt, 1986; Hayashi et al., 2005; Leca et al., 2011).

Tan (2017) applied a Perception-Action perspective theory to explain the development of stone-tool use behaviors of Koram *M. f. aurea* x *M. f. fascicularis* hybrid macaques. The infant macaques developed the behaviors by a simple manipulation with the objects involved in tools, e.g., stones and food items, followed by a nonfunctional combinatory manipulation and a subsequent stone-tool use behavior, playing before becoming skillful tool users. The hierarchical classification of manipulative bouts, based on the number of objects involved, the number of actions involved and the relations between objects, could illustrate the complexity of manipulative bouts changed through their development.

Since only *M. f. aurea* and *M. f. aurea* x *M. f. fascicularis* hybrids have been found using stones as tools to access encased food, it is interesting to know if genetics plays a role in the emergence of this behavior. This study surveyed the stone-associated behaviors (stone-tool use and stone-assisted behaviors) in *M. fascicularis* populations in the *M. f. aurea* x *M. f. fascicularis* intraspecific hybrid zone and vicinity, and assessed the association between the behaviors and the subspecies

identification (by both phenotypes and genetics). Following the results in Chapter III, the discordance of the subspecies identification based on phenotype and genetic (mtDNA & *SRY* sequence) analyses was observed, thus an association between behaviors and each of subspecies identification (based either on phenotype or genotype) was analyzed. As the 868 SNPs can clearly describe the degree of genetic admixture of *M. f. aurea* ancestry in the hybrid macaques reported in Chapter IV, this Chapter will also analyze the association between the degree of genetic admixture of *M. f. aurea* ancestry and behaviors in *M. f. aurea* and *M. f. aurea* x *M. f. fascicularisk* hybrid populations.

Methods

Ethical note

The permit for research and sample collection in Thailand was approved by the NRCT and the DNP. The IACUC of NPRCT-CU approved this study's experimental protocols (Protocol Review no. 1975007). The research adhered to the ASP Principles for the Ethical Treatment of Non-Human Primates.

Survey of stone-associated behaviors

The behaviors of 16 populations of *M. fascicularis* were surveyed and recorded (Table 5.1). First, the animals were observed if they performed stone-tool use or stone-assisted behaviors. If the stone tool-use behavior was not detected during the time of the survey, the evidence of the stone-tool use, such as broken pieces of oysters, snails, or crabs on the anvil rock with the stone tool left nearby, was surveyed. If neither the stone tool-use behavior nor the remnant of stone-tool use was

detected, the stone-tool test (Gumert, 2018) was performed. Regarding the stone tool test, in brief, the encased food objects that need materials to crack opened, e.g., clams (*Mercenaria* sp.) and mussels (*Perna sp*.), and hand-size stones that could be used as hammers were provided to monkeys. Those materials were placed on embedded boulders or hard substances such as concrete floors (Figure 5.2a). To avoid disturbing the daily activities of monkeys, the observer kept at least 3 m away from monkeys. The behaviors were categorized into four levels based on their complexity, from most complex to least complex (Tan, 2017); 3, 2, 1, and 0 (see Table 5.2) (Huffman, 1984; Nahallage and Huffman, 2008, Shumaker et al., 2011).

Subspecies status and degree of genetic admixture of *M. f. aurea* ancestry

Subspecies of *M. fascicularis* were identified as *M. f. aurea*, *M. f. fascicularis*, or *M. f. aurea* x *M. f. fascicularis* hybrid based on phenotypes (morphological subspecies) and mtDNA and *SRY* sequence analyses (genotypic subspecies) as mentioned in Chapter III. The degree of genetic admixture of *M. f. aurea* ancestry was estimated in 15 populationss using 868 RADseq-derived autosomal SNPs as mentioned in Chapter IV. However, only five out of 15 populations that the autosomal SNPs were analyzed, and the behavioral information was available and included in the analysis of this chapter. Morphological subspecies, genotypic subspecies based on mtDNA and *SRY* gene, and degree of genetic admixture *M. f aurea* ancestry in each *M. fascicularis* population and their behavioral levels are shown in Table 5.1.

Association of behavioral levels and subspecies identification

Apart from 16 populations of *M. fascicularis* that were surveyed in this study, the behavioral data of the other 10 populations were obtained from the previous studies (Bunlungsup et al., 2016; Ei Than, 2017). In total, 26 populations were included in the association analysis of behavioral levels and morphological subspecies, while only 22 populations (except JLI, LIL, ALP and KKN) could be identified for the genotypic subspecies and were recruited in the association analysis (Table 5.1). To assess the associations between behavioral levels and subspecies identification, the Kruskal-Wallis H test was first used to test if there were significant differences of behavioral levels among subspecies. The post-hoc pairwise comparisons were later analyzed the significant difference of behavioral levels in each pair of subspecies with adjusted p-value using Bonferroni correction. All analyses were performed in SPSS software version 28.0 (IBM Corp, 2021). Since the sample size for testing of an association between behavioral levels and degree of *M. f. aurea*'s genetic admixture was too small, no statistical test was performed, and only descriptive results were presented.

Table 5.1 Locality, GPS coordinates, morphological and genotypic subspecies based on mtDNA and *SRY* gene, percentage of genetic admixture of *Macaca fascicularis aurea* ancestry based on 868 RADseq-derived SNPs and levels of stone-associated behaviors in *Macaca fascicularis*.

	Locality	GPS (N, E)	Morphology	Genotype	*Mfa* admixture	Behavioral levels	Note
1.	Bang Taboon (BTB)	13°15', 99°56'	*Mff*	*Mff*	-	0	This study
2.	Wat khao thamon (WKT)	13°02', 99°57'	*Mff*	*Mff*	14	0	Bunlungsup et al. (2016)
3.	Wat Khao Takiab (WKTK)	12°30', 99°58'	Hybrid	*Mff*	-	1	This study
4.	Koram Island (KRI)	12°14', 100°00'	Hybrid	Hybrid	-	3	Bunlungsup et al. (2016)
5.	Lampi Island (LPI)	10°54', 98°12'	*Mfa*	*Mfa*	-	3	Bunlungsup et al. (2016)
6.	Ban Mai Somboon school (BMS)	10°51', 99°13'	*Mfa*	Hybrid	-	0	Bunlungsup et al. (2016)
7.	Jarlan Island (Lord Loughborough) (JLI)	10°25', 97°56'	*Mfa*	-	-	3	Bunlungsup et al. (2016)
8.	Thamprakayang (TPK)	10°19', 98°45'	Hybrid	*Mfa*	-	0	This study
9.	World War Museum (WWM)	10°10', 98°43'	*Mfa*	*Mfa*	99	0	This study
10.	Wat Paknam Pracharangsarith (WPN)	9°57', 98°35'	*Mfa*	*Mfa*	97	3	Bunlungsup et al. (2016)
11.	Mangrove Forest Research Center (MFRC)	9°52', 98°36'	*Mfa*	*Mfa*	97	2	Bunlungsup et al. (2016)
12.	Piak Nam Yai Island (PNY)	9°35', 98°28'	*Mfa*	*Mfa*	-	3	Bunlungsup et al. (2016)
13.	Lilet (LIL)	9°13', 99°14'	*Mff*	-	-	0	This study
14.	Check-In andaman pier (CIA)	8° 33', 98°13'	Hybrid	Hybrid	-	1	This study
15.	Wat Suwan Khuha (WSK)	8°25', 98°28'	*Mff*	*Mff*	7	0	Bunlungsup et al. (2016)
16.	Boi Noi island (BNI)	8°10', 98° 32'	Hybrid	*Mff*	-	3	This study
17.	Ao Lobi plantation (ALP)	8°10', 98°37'	Hybrid	-	-	3	This study
18.	Boi Yai Island (BYI)	8°07', 98°33'	Hybrid	*Mff*	-	3	This study
19.	Yao Yai island (YYI)	8°07', 98°31'	Hybrid	*Mff*	-	3	This study
20.	Sukha Pier (SKP)	8°06', 98°35'	Hybrid	*Mff*	-	2	This study
21.	Khao Kanab Nam (KKN)	8°04', 98°55'	*Mff*	-	-	0	This study
22.	Bang Rong pier (BRP)	8°02', 98°24'	Hybrid	Hybrid	-	0	This study
23.	King Kaew Soi Kao (KKSK)	7°54', 98°24'	Hybrid	Hybrid	-	2	This study
24.	Sirae Island (SRI)	7°53', 98°25'	Hybrid	Hybrid	-	3	Ei Than (2017)
25.	Baan Bor Rae (BBR)	7°50', 98°23'	Hybrid	Hybrid	-	2	This study
26.	Klong Mudong (KMD)	7°50', 98°22'	Hybrid	Hybrid	-	2	This study

Table 5.2 Four categories of the stone-associated behaviors (stone-tool use or stone-assisted behaviors) in *Macaca fascicularis* living at the *M. f. aurea* x *M. f. fascicularis* hybrid zone and vicinity.

Categorized behaviors	Levels	Definition
Stone-tool use	3	Monkeys use stones as tools to access encased foods.
Food pounding	2	Monkeys directly pound the encased foods with other hard substrates or pound to an unmanipulated hard substrate, including embedded stones.
Stone play	1	Monkeys manipulate stones, e.g., rolling, rubbing, or scratching, with no evident proximate (immediate and tangible) purposes.
None	0	None of the behaviors described above is detected. Note that the “none” populations with no stone tool test performed were not included in this study.

Result

Prevalence of stone-associated behaviors in *M. fascicularis*

Stone-associated behavior in each *M. fascicularis* population was shown in Table 5.1 and Figure 5.1. The stone-tool use behavior in BNI, BYI, YYI, and ALP populations reported previously (Luncz et al., 2017a; Gumert, 2018; Luncz et al., 2019) was confirmed in this study. Stone-tool use sites were along the intertidal zone in these locations (Figure 5.2b). Food pounding behavior was observed in SKP, KKSK, BBR, and KMD populations. They held clams in one hand and pounded to unmanipulated objects, e.g., embedded stones, wood, or mangrove roots (Figure 5.2e). Some monkeys held a clam on one hand and a manipulable object, e.g., glass bottle or other clams on the other hand and struck them together. Stone-play behavior was confirmed in WKTK population (Carter et al., 2016) and newly found in CIA population (Figure 5.2f). The WKTK monkeys rubbed stone on other stone or cement floor. When clams were provided, they also rubbed clams on stones or cement floor, or bit open them without any percussion. Some monkeys foraged and ate oysters attached to boulders in the intertidal region without using stone tools. Monkeys in the CIA population rubbed small stones on boulders embedded in the ground or on wrecked wood, but no percussive behavior was observed, though the clams were provided to the monkeys. Even if the stone tool test was performed, the BTB, TPK, WWM, LIL, KKN, and BRP monkeys have not performed any stone-associated behaviors. Most of the monkeys in these populations picked up clams, smelled, and then discarded them.

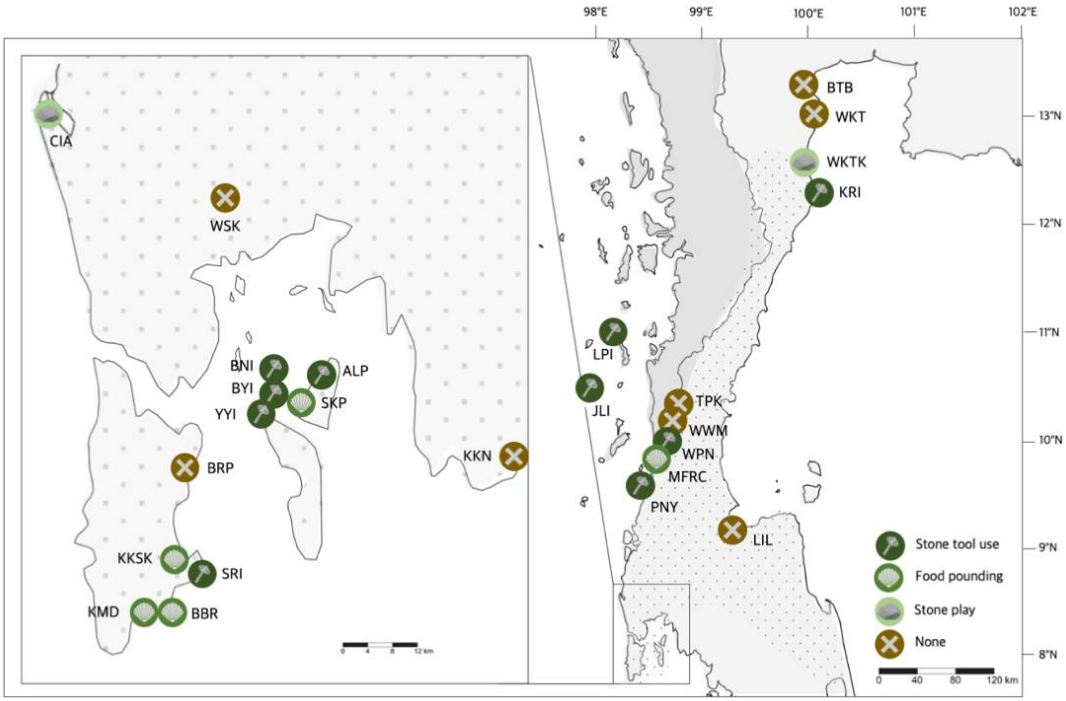


Figure 5.1 The distribution of stone-associated behaviors in *Macaca fascicularis* in Thailand. Three to four-letter codes are the name of populations corresponding to those in Table 5.1.

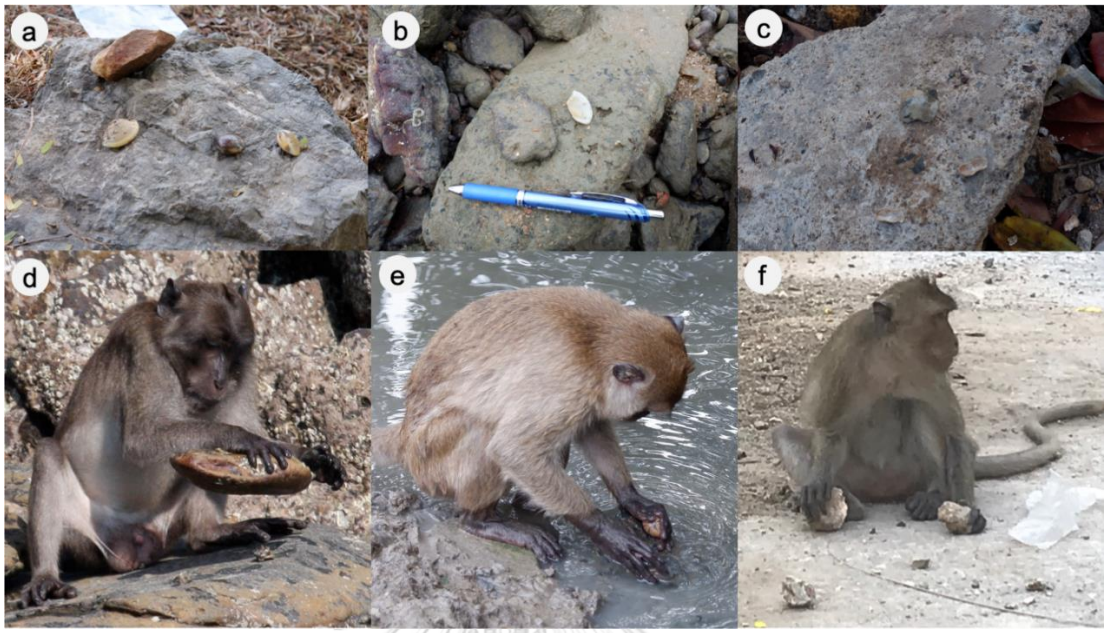


Figure 5.2 Behavioral sites and levels. (a) Stone-tool test site; clams and hand-size stone were placed on an embedded boulder. (b) Stone-tool use site; hammer stone and food debris were left on anvil at YYI. (c) Stone-play site; small stone was discarded on the boulder after CIA monkeys performed stone-play behavior. (d) Stone-tool use of *M. f. aurea* (Michael Gumert, 2011, with permission). (e) Food pounding; a monkey pounded a clam on an embedded stone at KMD. (f) Stone play; a monkey held stones in both hands and rubbed them onto the concrete floor at WKTK.

The association of the stone-associated behaviors and subspecies identification

The Kruskal-Wallis *H* test showed a statistically significant difference of behavioral levels across morphological subspecies (Kruskal-Wallis $H = 8.546$, p-value = 0.014), with a mean rank behavioral level of 5.00 for *M. f. fascicularis*, 15.43 for hybrid, and 15.71 for *M. f. aurea*. The post-hoc pairwise comparisons confirmed the significant differences between *M. f. fascicularis* and hybrids (adjusted p-value = 0.017) and between *M. f. fascicularis* and *M. f. aurea* (adjusted p-value = 0.034), while there was no significant difference between hybrids and *M. f. aurea* (adjusted p-value = 1.000).

Regarding the association between stone-associated behavior prevalence and genotypic (mtDNA and *SRY* gene) subspecies, the Kruskal-Wallis *H* test indicated no statistically significant difference in the distribution of behavioral levels across genotypic subspecies (Kruskal-Wallis $H = 0.256$, p-value = 0.880), with a mean rank of 11.00 for *M. f. fascicularis*, 11.19 for hybrid, and 12.58 for *M. f. aurea*.

Among five *M. fascicularis* populations (WKT, WWM, WPN, MFRC, and WSK) that the degree of genetic admixture of *M. f. aurea* ancestry was known, an association of the stone-associated behaviors and the degrees of genetic admixture of *M. f. aurea* ancestry can be drawn as follows. The WPN and MFRC populations had 97% genetic admixture, and performed stone-tool use and food pounding behaviors, respectively. The WWM, WKT, and WSK populations possessed 99%, 14%, and 7% of *M. f. aurea*'s genetic admixture, but they did not show any stone-associated behaviors.

Discussion

This study illustrated an association between stone-associated behaviors and morphological subspecies identification, but not with the genotypic subspecies identification based on mtDNA and *SRY* sequence analyses. It reflected the low sensitivity and limitation of uniparental markers in detecting the hybrid populations. The limitation of uniparental markers might be magnified in populations away from the hybrid zone since male monkeys had less opportunity to pass on their *M. f. aurea* Y-chromosome haplotypes to that population. Besides, some populations might have gone through the backcross reproduction with *M. f. fascicularis*, and the *M. f. aurea* Y-chromosome haplotypes might be diluted. However, in those *M. fascicularis* populations where the *M. f. aurea*'s genetics admixed in the autosomal genome can reflect through their morphology.

Stone play or stone manipulation behavior was proposed to be the precursor of stone-tool use behavior. As the stone play behavior was found to be accumulated in terms of diversity and complexity through generations, it potentially allowed the functional uses of stones to emerge (Huffman & Quiatt, 1986; Hayashi et al., 2005; Leca et al., 2011; Tan, 2017). This stone play behavior has been reported in *M. fascicularis*, *M. mulatta*, *M. fuscata*, and *M. cyclopis*, reflecting shared propensity of the behaviors across macaques in *fascicularis*/*mulatta* species group (Nahallage & Huffman, 2012; Pelletier et al., 2017). Although *M. f. fascicularis* in Bali, Indonesia was reported to perform a stone play behavior (Pelletier et al., 2017), the morphologically identified *M. f. fascicularis* in this study were not seen a stone play behavior while two morphologically identified hybrid populations; WKTK and CIA were found to perform. In this study, the stone play behavior was not reported in

either morphologically or genotypically identified *M. f aurea* populations. Nonetheless, it could be understood that *M. f. aurea* populations, at least those who showed stone-tool use behavior, performed stone play behavior during the stone-tool use developmental steps (Tan, 2017).

According to the description of Shumaker et al. (2011), some cases of food pounding, e.g., KMD monkeys that held clams in one hand and pounded on embedded stone, can be considered as proto-tool use since the object of change (clam), but not the agent of change (embedded stone), was manipulated, therefore the clam was not considered as tools. If this embedded stone functioned as moved or manipulated anvils, it is counted as proto tools. This study showed that food pounding behavior was only found in either morphologically identified hybrid or *M. f. aurea* populations but not in *M. f. fascicularis* populations. The absence of food pounding behavior in *M. f. fascicularis* populations may reflect the rarity of percussive actions in this subspecies.

Hayashi et al. (2005) proposed three possible causes that made chimpanzees fail to perform the nut-cracking behavior: the lack of hitting action, the scarcity of stone manipulation, and the difficulty of three object combinations. The experimental test of stone-tool use, i.e., pound-hammering behavior, in *M. f. fascicularis* showed similar possible causes. Bandini & Tennie (2018) indicated that naïve, captive *M. f. fascicularis* failed to learn individually, and socially to crack open encased food items (macadamia nuts). The macaques were found to rarely manipulate stones. While they manipulated food items in seven different patterns, only 3-4% of hit/drop pattern was performed. The results of Bandini & Tennie (2018) were concordant with the findings of this study that stone play and food pounding behaviors were found only in either

morphologically identified hybrid or *M. f. aurea* populations but not in *M. f. fasicularis* populations. The higher prevalence of stone play and food pounding behaviors in hybrids and *M. f. aurea* potentially reflects the inherited disposition that might influence the propensity of stone manipulation and percussive actions in *M. f. aurea* and its descendants (Nahallage & Huffman, 2012).

The statistically significant differences in stone-associated behavioral levels were detected between different morphologically identified subspecies; *M. f. fascicularis* and hybrid, and *M. f. fascicularis* and *M. f. aurea*. This indicated that *M. f. aurea* and hybrids had a higher prevalence of more complex stone-associated behaviors than the *M. f. fascicularis*. This finding is concordant with the report of Gumert et al. (2019), who categorized macaques within hybrid populations at Koram and Nom Sao islands, Khao Sam Roi Yot National Park, Prachuap Khiri Khan province into two groups based on their lateral facial crest patterns: hybrid-like macaques and *M. f. fascicularis*-like macaques. The study indicated a significantly higher prevalence of stone-tool users in hybrid-like macaques than in *M. f. fascicularis*-like macaques. This implies the propensity of the genetic effect on the stone-tool use behaviors because the monkeys inclusively lived in the same ecological and social conditions (Gumert et al., 2019). The significantly higher complexity of stone-assisted behaviors in *M. f aurea* and hybrids might reflect the higher cognitive abilities in these subspecies than in *M. f. fascicularis* (Heldstab et al., 2016).

Similarly to the previous studies denoted the genetic influences on cognitive abilities and development in humans (Hill et al., 2014; Lam et al., 2017; Mollon et al., 2021) and on tool-use performances in chimpanzees (Hopkins et al., 2015; Hopkins et al., 2016) the higher degree (97%) of genetic admixture of *M. f. aurea* ancestry was

detected in “stone-tool use” WPN population and “food pounding” MFRC population, while those of the lower degrees (14% and 7%) were detected in “none” WKT and WSK populations. This underpins the possibility of the genetic influences on the emergence, the prevalence, and the restriction of stone-tool use behavior in *M. f. aurea* and hybrids. Note, only five populations of *M. fascicularis* that the data of the degree of genetic admixture of *M. f. aurea* ancestry were available and included in this study. To further understand the degree of genetic admixture on stone-tool use and stone-assisted behavioral prevalence, more populations should be included for analysis, especially the populations that indicated the contradiction of morphological and genotypic (mtDNA and *SRY* gene) subspecies identification.

This preliminary investigation of stone-associated behaviors in *M. f. aurea*, *M. f. fascicularis*, and *M. f. aurea* x *M. f. fascicularis* hybrid populations in Thailand provided potential genetic propensity on the emergence and development of the stone-tool use behavior in *M. fascicularis*. Remarkably, Laland & Janik (2006) stated that neither genetic, ecological, or cultural variations could solely explain the tool-use variations across the animal kingdom. It is probably all three factors integrated. Therefore, ecological factors (i.e., habitat use and stone availability) and social-culture factors (i.e., food preference and social tolerance) should be considered.

To elucidate if the *M. f. aurea*’s genetic proprietary influences on stone-tool use behavior, the further work in macaque population (s) that share the same ecological and social-culture factors should be done. The study of *M. f. aurea* genetic admixture at an individual level in that *M. f. aurea* x *M. f. fascicularis* hybrid population should potentially help to illustrate the *M. f. aurea* genetic factor on the stone-tool use behavior. The Koram island hybrid population at Khao Sam Roi Yot National Park,

Prachuap Khiri Khan province, that was previously reported the different prevalences of stone-tool use behavior between the two morphological groups of hybrid macaques (Gumert et al., 2019), would probably be a good choice.

CHAPTER VI
GENERAL DISCUSSION AND CONCLUSION

M. f. aurea, the only stone-tool user of Old World monkeys (Luncz et al., 2019), was proposed to be originated from the ancient hybridization between proto-*M. f. aurea* males and *sinica* species in modern-day Myanmar or Bangladesh during 2.58 – 0.95 MYA (Matsudaira et al., 2018). The mainland Myanmar *M. f. aurea* that carried the oldest mtDNA haplotype supported the hypothesis of their origin in Myanmar (Figure 3.2, Bunlungsup et al., 2016; Matsidaira et al., 2018). *M. f. aurea* have expanded their distribution from mainland Myanmar along the Andaman Sea Coast and occupied the Mergui Archipelago and southwestern Thailand in the region of Ranong province (9°35'N - 10°19'N) (Fooden 1995; San & Hamada, 2011; Bunlungsup et al., 2016). This expansion tended to occur during 0.99 – 0.37 MYA after the split of mainland Myanmar *M. f. aurea* lineage and before the diversification of the Mergui Archipelago and Thailand *M. f. aurea* populations (Figure 3.5, Table 3.2). That was potentially induced by the influences of EMPT which was well-known for the increase in glacial-interglacial cycle interval and the high amplitude of climate oscillations (Clark et al., 2006; Head & Gibbard, 2015; Chalk et al., 2017) which contributed to terrestrial biota composition (Head & Gibbard, 2015), genetic consequences of organisms (Hewitt, 2004; Othman et al., 2020; Ghane-Ameleh et al., 2021), and migration of animal species including primates (Head & Gibbard, 2015).

The intraspecific hybridization between *M. f. aurea* and *M. f. fascicularis*, based on morphological subspecies identification (Table 3.1), was found ranging from 7°50'N - 12°30'N in the peninsula part of Thailand, which was slightly broader than

that in the previous reports (Fooden, 1995; Bunlungsup et al., 2016). The genetic analyses of partial mtDNA (674 bp) and two Y-chromosome genes (*SRY* and *TSPY*, 184 and 2039 bp, respectively) confirmed the male-mediated hybridization from *M. f. aurea* to *M. f. fascicularis* in two different migration routes: southward and northern-eastward routes, which were aligned with the *TSPY* haplotype network and distribution pattern (Figure 3.2, 3.3, 3.4; Bunlungsup et al., 2016). The analysis of 868 RADseq-derived autosomal SNPs supports an asymmetric (unidirectional) introgression from *M. f. aurea* to *M. f. fascicularis* populations because a higher proportion of *M. f. aurea* ancestry in *M. f. fascicularis* individuals (up to 14%) than in the opposite direction (up to 2%) was detected. It also illustrated the genetic introgression of *M. f. aurea* that extended beyond the intraspecific hybrid zone based on morphology and mtDNA and Y-chromosome gene sequence analyses (Figure 4.1, 4.2). This is the first study reporting the possibility of genetic introgression of *M. f. aurea* into Indochinese *M. f. fascicularis* located in central Thailand (TST; 15°56'N, 99°57'E), which is approximately 500 km north of the previously proposed intraspecific hybridization zone (8°10'-12°24'N; Fooden, 1995; Bunlungsup et al., 2016).

The distribution of *M. f. aurea* x *M. f. fascicularis* hybrid populations, either based on morphology, mtDNA and Y-chromosome gene sequences, or autosomal SNPs, seemed restricted in Indochina and Sunda regions in the western side of the peninsula Thailand. This illustrated the influences of the Phuket Range (9°20N, 98°37′E), the Nakhon Si Thammarat Range (8°30′N, 99°34′E), and the Surat Thani-Krabi depression that might be a zoogeographical barrier for the migration/introgression of *M. f. aurea* to the Sundaic *M. f. fascicularis* populations on

the eastern side. The contribution of these zoogeographical barriers has been reported previously in the population history of Sundaic *M. f. fascicularis* (Bunlungsup et al., 2017a) and the distribution limit of northern (*M. leonina*) and southern pig-tailed macaques (*M. nemestrina*) (Malaivijitnond et al., 2012).

M. f. aurea and hybrids between *M. f. aurea* and *M. f. fascicularis* have been reported to habitually use stones to process various types of encased foods (Gumert and Malaivijitnond, 2012; Bunlungsup et al., 2016; Tan, 2017; Lunzc et al., 2017) while it has never been reported in *M. f. fascicularis* (Malaivijitnond & Hamada, 2008; Bandini & Tennie, 2018). Nonetheless, this study indicated the incongruence of morphologically and genotypically (mtDNA and *SRY*) identified subspecies. The statistically significant differences in stone-associated behavioral levels were detected between different morphologically identified subspecies; *M. f. fascicularis* and hybrid, and *M. f. fascicularis* and *M. f. aurea*. This indicated that *M. f. aurea* and hybrids had a higher prevalence of more complex stone-associated behaviors than the *M. f. fascicularis,* which is concordant with the report of Gumert et al. (2019). The stone-tool use, and food-pounding populations had a higher degree (97%) of genetic admixture of *M. f. aurea* ancestry. In comparison, the populations without any stone-associated behaviors showed the lower degrees (14% and 7%) of *M. f. aurea* ancestry. The findings of this study illustrated the propensity of the genetic effects on the stone-tool use behaviors. The significantly higher complexity of stone-associated behaviors in *M. f aurea* and hybrids might reflect the higher cognitive abilities in these subspecies than in *M. f. fascicularis* (Heldstab et al., 2016). Similarly, the previous studies denoted the genetic influences on human cognitive abilities and development (Hill et al., 2014; Lam et al., 2017; Mollon et al., 2021) and on tool-use performances

in chimpanzees (Hopkins et al., 2015; 2019). This underpins the possibility of the genetic influences on the emergence, the prevalence, and the restriction of stone-tool use behavior in *M. f. aurea* and hybrids. Remarkably, Laland & Janik (2006) stated that neither genetic, ecological, or cultural variations could solely explain the tool-use variations across the animal kingdom; it is probably all three factors integrated. Therefore, ecological factors (i.e., habitat use and stone availability) and social-culture factors (i.e., food preference and social tolerance) should be considered for future studies.

M. f. aurea is a critical taxon that helps fulfilling the knowledge of stone-tool use behavior/cognitive development in primate species (Gumert et al., 2009; Gumert et al., 2011; Gumert & Malaivivjitnond, 2012; Gumert &Malaivijitnond, 2013; Falótico et al., 2016; Haslam et al., 2016). Their natural distribution in Thailand occurs in islands and coastal regions with rocky shore, or mangrove habitats in only Ranong province (9°35'N - 10°19'N). The mangrove deforestation for agriculture and aquaculture has increased in southern Thailand (Pumijumnong, 2014; Richards & Friess, 2016). This threat can lead to *M. f. aurea*'s natural habitat loss and the population reduction (Kabir & Ahsan 2012; San and Hamada, 2011). Although long-tailed macaques have been recognized as the greatly adaptive non-human primates and considered as pest species in some areas (San & Hamada, 2011), the natural habitat loss and the more anthropogenic activities involved may lead to the loss of natural behaviors of *M. f. aurea* including stone-tool use behaviors. This would be even worse if the loss of the population itself is happened as seen in *M. f. aurea* in Bangladesh recently. Therefore, the authorities should seriously consider setting up a strategy to conserve the habitat together with the natural behaviors of *M. f. aurea*.

The genetic uniqueness of *M. f. aurea* is indicated in the divergence of *M. f. aurea* mtDNA from *M. f. fascicularis*/*M. mullata* mtDNA (Figure 3.2, Bunlungsup et al., 2016) and grouped with *sinica* species group in the mtDNA phylogenetic analysis (Matsudaira et al., 2018; Osada et al., 2021). The autosomal SNP analyses emphasized the genetic difference of *M. f. aurea* from the conspecific *M. f. fascicularis* and pronounced the two geographical forms of *M. f. fascicularis*; Indochinese and Sundaic forms (Figure 4.2, 4.3; Osada et al., 2021).

Apart from the *M. mulatta*, *M. fascicularis* is one of the most commonly used non-human primate species in biomedical research (Bonhomme et al., 2009). The genetic background, geographic origin, and proportions of inter and intraspecific admixture of the animals that potentially affect results (e.g., disease susceptibility, drug metabolism, and vaccine responses) (Stahl-Hennig et al., 2007; Degenhardt et al., 2009; Liao et al., 2012; Zhang et al., 2017; Uno et al., 2018) should be considered. The genetic difference between *M. f. fascicularis* and *M. f. aurea* is as significant as between Indian and Chinese *M. mulatta,* which are thought to belong to separate subspecies (Ferguson et al., 2007; Hernandez et al., 2007; Malhi et al., 2007). Of great biomedical relevance, the Indian *M. mulatta* is often considered to be a more suitable model for HIV research than Chinese *M. mulatta* because the Indian-origin animals develop SIV symptoms that mirror those of humans with AIDS in terms of disease patterns and incidence and severity (Cohen, 2002). Similarly, the genetic differences between *M. f. aurea* and *M. f. fascicularis* could also render certain subspecies over others like more appropriate model for specific biomedical studies, such as cognitive science (Gumert and Malaivijitnond, 2013; Bandini and Tennie, 2018).

In conclusion, this study illustrated the genetic diversity, admixture, and complexity of the genetic make-up of *M. fascicularis* in Thailand, where the core hybridization between the two species (*M. fascicularis* and *M. mulatta*) and the two subspecies (*M. f. fascicularis* and *M. f. aurea*) occurred. This can provide basic information to help minimize errors in interpreting experimental outcomes when using *M. fascicularis* as animal models in biomedical research (Zhang et al., 2017; Li et al., 2020) and promote the reduction in the number of animals used for experimentation following the principles of the 3Rs (replacement, reduction, and refinement; Haus et al., 2014). The differences of stone-associated behaviors across *M. fascicularis* populations with different degrees of *M. f. aurea* ancestry could potentially be the best model in studying the genetic influences on the variation of cognitive abilities in *M. fascicularis* and other primate species.

Executive summary

The present work studied the genetic characteristics of *M. f. aurea*, *M. f. fasicularis,* and *M. f. aurea* x *M. f. fascicularis* hybrids in Thailand by using mtDNA, Y-chromosome genes (*SRY* and *TSPY*), and RADseq-derived autosomal SNPs. It illustrated the influence of the early-middle Pleistocene transition on the linage split and migration of *M. f. aurea,* and an asymmetrically genetic introgression of *M. f. aurea* to *M. f. fascicularis* populations that occurred far beyond the previously reported intraspecific hybrid zone. The statistically significant association of the morphologically identified *M. f. aurea* and the hybrids with the stone-associated behaviors, and the tendency of exhibiting the more complex stone-associated behaviors in the macaque populations with the higher degree of *M. f. aurea* ancestry

partially implied the genetic influences on the emergence, the prevalence and the restriction of stone-tool use behavior in *M. f. aurea* and hybrids.

Perspective/ Future work

This study included *M. f. aurea* populations in Thailand, Mergui Archipelago and only one population locating in northern distribution region in mainland Myanmar. To fulfil the understanding of *M. f. aurea* origin, migration, stone-tool use emergence and distribution, future studies might have to include more populations from Myanmar which should cover all of their natural distribution. The genetic admixture analysis indicated the low percentages of *M. mulatta* ancestry occurred in the *M. f. aurea* mainland Myanmar population, comparing to those occurred in *M. f. fascicularis* populations. The explanations why the degree of *M. mulatta* genetic introgression into *M. f. aurea* and *M. f. fascicularis* are so different are needed.

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VITA

NAME	Poompat Phadphon
DATE OF BIRTH	11 September 1994
PLACE OF BIRTH	Ubonratchathani, Thailand.
INSTITUTIONS ATTENDED	Master of Science Program in Zoology at Department of Biology, Faculty of Science, Chulalongkorn University.
HOME ADDRESS	404 Moo. 11, Napho subdistrict, Buntharik district, Ubonratchathani province, Thailand, 34230.
PUBLICATION	Phadphon P, Kanthaswamy S, Oldt RF, et al. (2022). Population structure of Macaca fascicularis aurea, and their genetic relationships with M. f. fascicularis and M. mulatta determined by 868 RADseq-derived autosomal SNPs-A consideration for biomedical research. J Med Primatol, 51(368), 33-44.
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	5-month Master research supported by DPST project, Thai government scholarship, at Arizona State University West Campus, Arizona, USA, under supervision of Professor Dr. Sree Kanthaswamy.