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RESEARCH ARTICLE

# Morphological Characteristics and Genetic Diversity of Burmese Long-Tailed Macaques (*Macaca fascicularis aurea*)

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*Macaca fascicularis aurea* (*Mfa*) is the only macaque which has been recorded to use stone tools to access encased foods. They live in close contact with *M. fascicularis fascicularis* (*Mff*) in southwestern Thailand and the hybrids were reported [Fooden, 1995]. Although *Mff* and *Mfa* can be seen in the same habitat types, tool-use behavior has never been reported in *Mff*. Thus, comparing the morphological characteristics and genetics between *Mfa* and *Mff* should help elucidate not only the morphological differences and genetic divergence between these subspecies but also potentially the relationship between genetics and their tool use behavior. We surveyed *Mfa* and *Mff* in Myanmar and Thailand, ranging from 16° 58′ to 7° 12′ N. Fecal or blood samples were collected from eight, five, and four populations of *Mfa*, *Mff*, and *Mff* × *Mfa* morphological hybrids along with three individuals of captive Chinese *M. mulatta* (*Mm*), respectively, for mtDNA and Y-chromosome (TSPY and SRY genes) DNA sequence analyses. In addition, eight populations were captured and measured for 38 somatometric dimensions. Comparison of the somatic measurements revealed that *Mfa* had a statistically significantly shorter tail than *Mff* ($P < 0.05$). Based on the mtDNA sequences, *Mfa* was separated from the *Mm*/*Mff* clade. Within the *Mfa* clade, the mainland Myanmar population was separate from the Mergui Archipelago and Thailand Andaman seacoast populations. All the morphological hybrids had the *Mff* mtDNA haplotype. Based on the Y-chromosome sequences, the three major clades of *Mm*/Indochinese *Mff*, Sundaic *Mff*, and *Mfa* were constructed. The hybrid populations grouped either with the *Mm*/Indochinese *Mff* or with the *Mfa*. Regarding the genetic analysis, one subspecies hybrid population in Thailand (KRI) elicited tool use behavior, thus the potential role of genetics in tool use behavior is raised in addition to the environmental force, morphological suitability, and cognitive capability. Am. J. Primatol. 

**Key words: Burmese long-tailed macaque; Andaman seacoast; mtDNA; TSPY; SRY**

## INTRODUCTION

Long-tailed or cynomolgus macaques (*Macaca fascicularis*) are the second most widely distributed and diversified macaque (after rhesus macaques [*Macaca mulatta*]), being found in a geographic area that encompasses continental and insular populations, which lead to their high genetic diversity [Fooden, 1995]. They have been classified into 10 subspecies based on their different geographic origins [Fooden, 1995]. The large number of studies focused on *M. fascicularis fascicularis* (*Mff*) is attributable to the much more widespread distribution of that subspecies than all others [Abdul-Latiff et al., 2014; Blancher et al., 2008; Li et al., 2012; Kanthaswamy et al., 2013; Tosi & Coke, 2007]. Only one genetic study of *M. fascicularis philippinensis* is currently available [Smith et al., 2014].

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*Macaca fascicularis aurea* (*Mfa*), one of the 10 subspecies of long-tailed macaques, is listed as data deficient for International Union for Conservation of Nature (IUCN). This subspecies is strikingly important and becomes a focal point of interest because it is the only one of the three non-human primate species that has been reported to use stone-tools to access protected food items, such as oysters [Gumert et al., 2009, 2011, 2013; Gumert & Malaivijitnond, 2012, 2013; Malaivijitnond et al., 2007]. Although the genetic variability of *Mfa* was recently assessed using microsatellite markers, those monkeys were known to originate from Cambodia [Li et al., 2012], which is not within the habitat range of *Mfa*. Rather *Mfa* lives mainly in Myanmar, which leads to their common name of Burmese long-tailed macaques, and is distributed southeastward along the Andaman seacoast through the Mergui Archipelago and western Thailand [Fooden, 1995; Malaivijitnond & Hamada, 2008; San & Hamada, 2011]. The major threats to *Mfa* populations are habitat fragmentation, encroachment of their mangrove forest habitats for shrimp culture and agriculture practices, hunting for food and trade [Gumert et al., 2013; Kabir & Ahsan, 2012; San & Hamada, 2011]. *Mfa* lives in close contact with *M. mulatta* (*Mm*) in central Myanmar and with *Mff* in southwestern Thailand at the vicinity of the Isthmus of Kra (10° 15′ N, 99° 30′ E). Hybrids between the different species (*Mfa* × *Mm*) or different subspecies (*Mfa* × *Mff*) have been reported or proposed [Fooden, 1995; Hamada et al., 2006]. Although *Mff* occurs parapatrically with *Mfa* and can be seen in the same habitat types, tool-use behavior has never been reported in *Mff* including in a 25-year field survey [Malaivijitnond & Hamada, 2008; Malaivijitnond et al., 2011].

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

With respect to the Isthmus of Kra zoogeographical barrier, *Mff* from the north and south of the Isthmus of Kra were morphologically [Hamada et al., 2008] and genetically different [Tosi et al., 2002], and separated into two forms (Indochinese and Sundaic). Basically, the Sundaic *Mff* form had a longer tail and smaller contrast of the yellow pelage color between the back and the thigh [Hamada et al., 2008]. One possible reason to explain these differences is that the *Mff* inhabiting north of the Isthmus of Kra are hybrids derived from the introgression of *Mm* males [Bonhomme et al., 2009; Kanthaswamy et al., 2008, 2010; Osada et al., 2010; Satkoski Trask et al., 2013; Stevison & Kohn, 2009; Tosi et al., 2002; Yan et al., 2011]. As such, some morphological characters of *Mm* should be transmitted into the Indochinese *Mff* populations. Regarding the genetic admixture of *Mm*, the hybrids between *Mfa* × *Mff* at the north and the south of the Isthmus of Kra [Fooden, 1995] should exhibit some differences in their morphological characters, at least for the pelage color, relative tail length, and cheek hair pattern.

So far, up to five geographic populations of *M. fascicularis* have been recorded to use tools; one each at the islands in the Mergui Archipelago of southern Myanmar [Carpenter, 1887], the Piak Nam Yai Island, Baan Koh Lao Island, Pracharatrangsarith Temple [Gumert et al., 2009; Malaivijitnond et al., 2007] and the Koram Island of Thailand [Aiempichitkijkarn et al., 2014]. All these populations were comprised of the *Mfa* subspecies except at Koram Island (KRI; the only population in the Thai Gulf), where the monkeys appeared by cheek hair pattern to be *Mfa* × *Mff* hybrids. However, these tool using macaques all lived in similar habitat types of islands or fringes of mangrove forests with encased marine invertebrate food and the availability of stone tools. There has been no report of tool use behavior in other mainland dwelling *Mfa* [San & Hamada, 2011]. Thus, comparing the morphological characteristics and genetics between *Mfa* and *Mff* should help elucidate not only the morphological and genetic differences between these subspecies, but also potentially the relationship between genetics and their tool use behavior.

## METHODS

### Field Survey of *Mfa*

The survey of *Mfa* was based on the information gathered from questionnaires [Malaivijitnond et al., 2011], interviews, gazettes, and published scientific reports in 2005–2014. We did survey in Myanmar, Mergui Archipelago, and Thailand covering the habitat range of *Mfa* [Fooden, 1995], except in Bangladesh. When we encountered the monkeys, we first identified the species and subspecies of monkeys or hybrids with regard to their morphological characters, mainly their cheek hair pattern, vertex of head crest, and pelage color [Fooden, 1995; Hamada et al., 2006, 2008]. It is difficult to classify subspecies by body proportion. We also looked for tool-use behavior. If it was feasible to gain access into their habitats, photographs and fecal samples were taken. In some

study sites (where feasible) monkeys were captured using a net or automatic box trap and anesthetized with either ketamine hydrochloride (Ketalar; Sankyo Co., Ltd.) at 10 mg/kg body mass or a mixture of ketamine hydrochloride (3 mg/kg body mass) and medetomidine (0.1 mg/kg body mass). The anesthetized subjects were then measured for 38 somatometric dimensions (Table SI) and pelage color, and blood samples were collected. After all the procedures were performed and complete consciousness of the monkeys was confirmed, they were returned to their respective habitats. The monkeys anesthetized with the ketamine hydrochloride and medetomidine mixture were antisedated with atipamezole ($\leq$0.1 mg/kg body mass) before being released.

The surveys in Myanmar were conducted according to the regulations of the Yangon University and the Ministry of Forestry of Myanmar, whereas in Thailand, it was permitted by the National Research Council of Thailand and the Department of the National Parks, Wildlife and Plant Conservation of Thailand. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Faculty of Science in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University, Protocol Review No. 1423010. This research adhered to the American Society of Primatologists principles for the ethical treatment of primates.

## Sample Collection and DNA Extraction

Blood or fecal samples of free ranging *Mfa*, *Mff*, and hybrids from eight, five, and four populations, respectively, were collected (Table I and Fig. 1). The eight populations of *Mfa* covered their distribution range, except for in southern Bangladesh [Fooden, 1995], because the populations previously reported in southern Bangladesh possibly no longer exist [Kabir & Ahsan, 2012], whereas the five populations of *Mff* lived parapatrically with *Mfa* in the Indochina and Sunda regions, where the four populations of morphological hybrids were attained. In addition, *Mm* derived from China and reared at the Primate Research Institute of Kyoto University, Japan, was included in the analysis, although *Mm* and *M. fascicularis* are classified in a same species group of *fascicularis* [Fooden, 1980] and it was reported about a genetic admixture between *Mm* and *M. fascicularis* by the male-mediated nuclear gene flow from the Chinese rhesus macaques to the Indochinese long-tailed macaques [Tosi et al., 2002].

After collection, the blood samples were centrifuged at 1,000*g*, 4°C for 10 min, and the buffy coats containing white blood cells were separated as reported previously [Malaivijitnond et al., 2007; Malaivijitnond & Hamada, 2008]. Genomic DNA from the buffy coats was extracted by the classical phenol–chloroform method [Sambrook et al.,

**TABLE I. Region, Locality, Geographical Coordinate, Morphological Species Identification (or Taxon), Collected Tissue Specimen and Tool Use in *Macaca fascicularis fascicularis* (*Mff*), *M. fascicularis aurea* (*Mfa*), and Their Hybrids (Bold Letters)**

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

Subspecies were identified based on their morphological characters [Fooden, 1995; Hamada et al., 2008].
[a]These eight groups were captured, their somatometric dimensions and pelage color were recorded, and blood samples were collected.
[b]This specimen was a pet macaque, which had been captured from Zadetkyi.

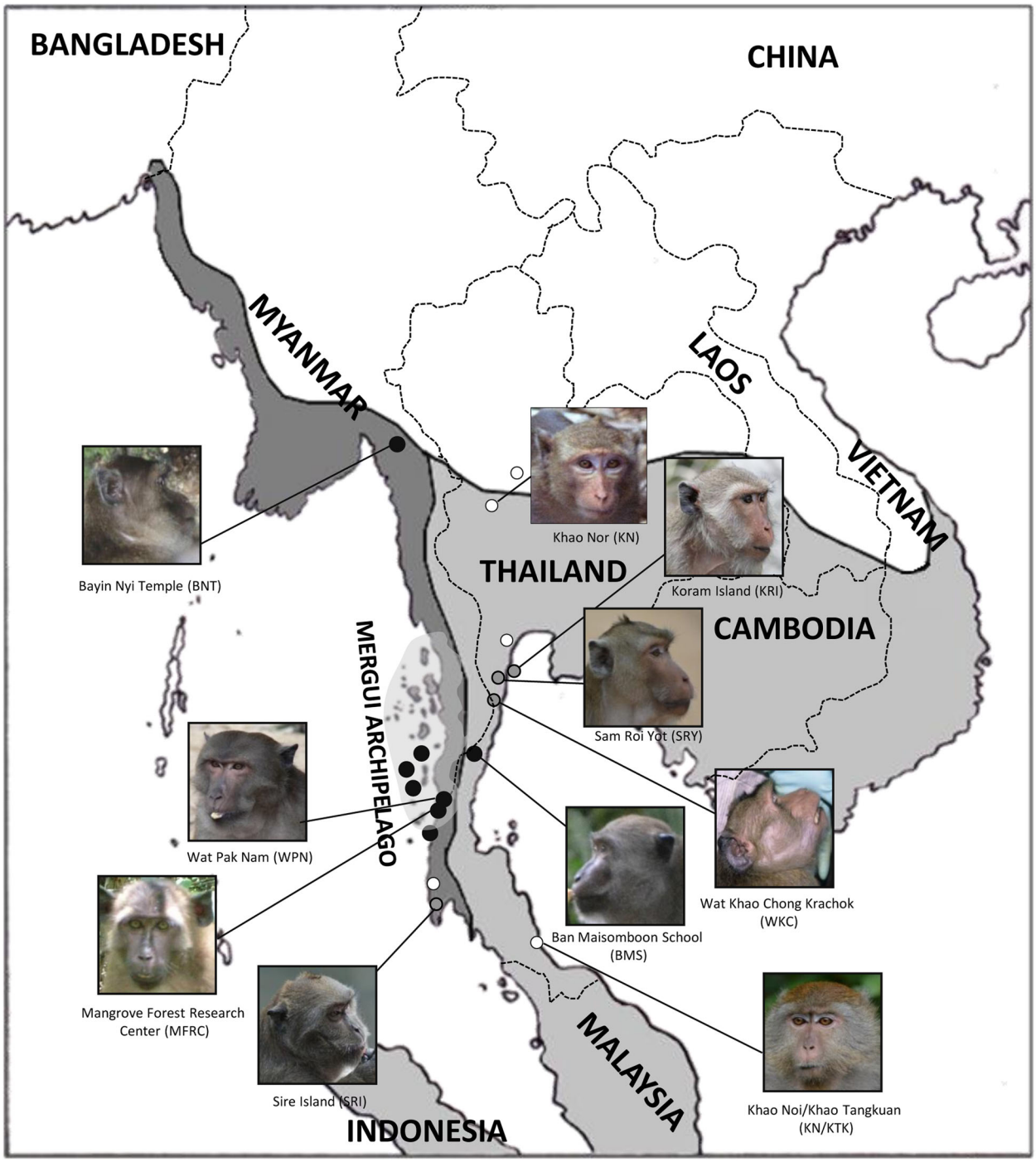


Fig. 1. Distribution range of *Macaca fascicularis aurea* (*Mfa*; black) and *M. fascicularis fascicularis* (*Mff*; gray). Black, white, and gray circles indicate the *Mfa*, *Mff*, and hybrid locations of the samples collected in this study.

1989] and kept at the DNA bank at the Primate Research Unit, Chulalongkorn University.

Epithelial cells from the digestive tract were harvested from the surface of fecal samples using a cotton swab and stored in 2 mL of lysis buffer (0.5% (w/v) SDS, 100 mM EDTA pH 8.0, 100 mM Tris–HCl pH 8.0 and 10 mM NaCl) at room temperature [Hayashi & Kawamoto, 2006]. DNA was then extracted using the QIAamp DNA Stool mini kit (QIAGEN Inc., Hilden, Germany) as per the manufacturer's protocol. The extracted DNA was used for PCR amplification and sequencing of the selected mitochondrial DNA (mtDNA) region and two Y-chromosome gene sequences (see below).

### Mitochondrial DNA and Y-Chromosome Gene Amplification and Sequencing

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

To trace the paternal inheritance, migration, and introgression pattern of the males, the sex-determining region Y-chromosome (SRY) and testis-specific protein Y-chromosome (TSPY) genes, which are approximately 800 bp and 2.3 kbp in length, respectively, were amplified. These two loci are located on the non-recombinant portion and mapped onto the short and long-arm of Y-chromosome, respectively. The SRY gene was amplified using the SW2/SW3B primer pair (Table II) as described by Whitfield et al. [1993] except with a slight modification to the annealing temperature (see below). The TSPY gene was amplified from the blood DNA samples using the TSPY-A/TSPY-5R primer pair (Table II) following Tosi et al. [2000]. However, due to the degradation of the fecal DNA samples, three pairs of primers (TSPY-A/TSR1012, TSF566/TSR1676, and TSF1383/TSPY-5R for 1012, 1110, and 855 bp of sequence, respectively) were used for amplification to cover the whole length of targeted TSPY gene as shown in Table II and Figure 2.

PCR mixtures (10 μL total) contained 0.625 U ExTaq DNA Polymerase (Takara Bio Inc., Shiga, Japan), 0.2 μM each primer and 50–100 ng DNA template in the manufacturer's buffer. In addition, for the extracted DNA from fecal samples, 0.12 μg T4 gene 32 protein (Wako Nippon Gene, Japan) was included to promote the DNA synthesis by DNA polymerase. The mtDNA amplification was performed with 40 cycles of 94°C for 25 sec, 63 to 60°C (decreasing at −0.1°C/cycle) for 30 sec and 72°C for 20 sec, and then followed by 72°C for 7 min. The SRY gene amplification was performed with 40 cycles of 94°C for 30 sec, 52–57°C for 20 sec, and 72°C for 2 min, followed by 72°C for 7 min. The TSPY gene amplification from blood DNA samples was performed with 45 cycles of 94°C for 25 sec, 66°C for 45 sec, and 72 °C for 3 min, followed by a final 72°C for 7 min. Due to the long length of the TSPY gene, the amplification from fecal DNA was performed with three pairs of primers and various annealing temperatures (Table II and Fig. 2) with a reduced extension time to 1 min.

All PCR reaction products were resolved and visualized on 1% (w/v) agarose gel-TAE electrophoresis followed by ethidium bromide staining and UV-transillumination. Specific PCR products were directly purified using the ExoSAP-IT kit (Affymetrix Inc., CA). Sequencing reactions were performed on both strands using the same PCR primers (individually), except the TSPY gene long amplicon acquired from the blood DNA was sequenced additionally with the TSF566 and TSR1676 internal primers (Table II). These internal primers could provide the partially overlapping region of sequences which was useful for sequence assembly. Sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3130*xL* Genetic Analyzer (Applied Biosystems, CA). Data output was assembled and analyzed on SeqMan$^{tm}$ II (DNASTAR Inc.) and Finch TV (Geospiza Inc., WA).

**TABLE II. Primer and Annealing Temperature for Each Marker Used in This Study**

| Primer | Nucleotide sequence (5′ to 3′) | Annealing temperature |
|---|---|---|
| HVSI primers | | |
| HVS-F | CCGCCCACTCAGCCAATTCCTGTTCT | 60–63°C |
| HVS-R | CCCGTGATCCATCGAGATGTCTT | |
| SRY primers | | |
| SW2 | CTTGAGAATACATTGTCAGGG | 52–57°C |
| SW3B | AGGTCTTTGTAGCCAATGTTACCCG | |
| TSPY primers (blood sample) | | |
| TSPY-A | AGCCAGGAAGGCCTTTTCTCG | 66°C |
| TSPY-5R | CTGTGCATAAGACCATGCTGAG | |
| TSPY primers (fecal sample) | | |
| TSPY-A | AGCCAGGAAGGCCTTTTCTCG | 66–68°C |
| TSR1012 | TGTCACCTGTGACGTTCACGA | |
| TSF566 | AGGTCATTCATGGATGCAGAT | 62–65°C |
| TSR1676 | CCACAGTTATAACCTGCTTTGC | |
| TSF1383 | AATCCCCTGCAATACTACAGGAGG | 62–66°C |
| TSPY-5R | CTGTGCATAAGACCATGCTGAG | |

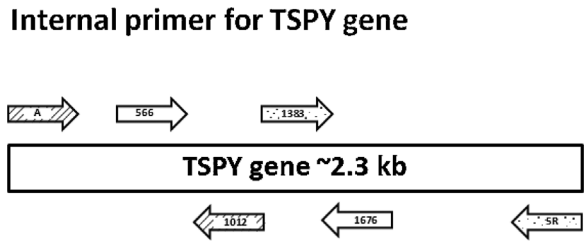


Fig. 2. Internal primers used in this study for the PCR amplification of the TSPY gene.

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

## Data Analysis and Phylogenetic Tree Construction

Although SRY and TSPY genes are linked and no conflict between the tree topologies was found between the SRY and TSPY data set when the partition homogeneity was tested by PAUP 4.0 program with 1,000 bootstrap [$P = 1.0$, Swofford, 2003], they were combined (concatenated) to one sequence containing 2,040 bp of TSPY and 686 bp of SRY for phylogenetic analysis [Tosi et al., 2000, 2002; Tosi & Coke, 2007]. The corresponding DNA sequences from *M. sylvanus* (Barbary macaque) were downloaded from Genbank and used as the outgroup (Accession numbers: NC_002764 for mtDNA, and AF284326, and AF284275 for the SRY and TSPY genes, respectively). The SRY and TSPY sequences of the Sundaic *Mff* from Indonesia (Java) and Malaysia (Selangor and Johor) (Accession numbers: AF425293, AF425294, AF425292, and AF284303 for SRY and AF425278, AF425279, AF425277, and AF284252 for TSPY) were also downloaded and included in the analysis to improve the resolution of any hybridization between *Mfa* and *Mff*. All DNA sequences were aligned by ClustalW as implemented in the Mega5.2 software [Tamura et al., 2011]. To include all sequences into the phylogenetic analysis both ends were trimmed off before the analysis was performed. Thus, the total of 677 and 2,726 bp of mtDNA and Y-chromosome gene, respectively, were used for analysis. The phylogenetic trees were constructed using distance-base method (Neighbor Joining). DNA sequences were checked for any indels using Mega 5.2 software. The phylogenetic tree was constructed using the Neighbor joining distance-based method (NJ), as implemented in the Mega5.2 software [Tamura et al., 2011] with 1,000 bootstrap.

## Somatometry and Pelage Color Determination

After the monkeys were anesthetized and collected blood samples, 38 somatometric dimensions in 144 adults and the pelage color in 267 individuals (all, except babies) of eight populations (WHM, WKT, WSK, and KN/KTK for *Mff*; WPN, MFRC, and BNT for *Mfa*; and WKC for hybrids; Table III) were measured. Although most of morphological datasets of Indochinese and Sundaic *Mff* were obtained from Hamada et al. [2008], those of *Mfa* were newly collected for this study. The seven proportions of the relative tail length (against the crown-rump length), fore/rear limb length ratio, hand width/length ratio, relative third finger length (against hand length), head width/length ratio, relative facial length ([upper facial height]/[head length]), and facial width/length ratio ([bizygomaitc breadth]/[facial height]) were calculated. Because not all of animals mentioned above could be collected these seven proportions, only 66, 26, 17, and 21 of Indochinese *Mff*, Sundaic *Mff*, hybrid, and *Mfa* were used for the analysis (Table IV). The dental eruption pattern of the animals was also recorded and used for the age estimation based on the norm established by Smith et al. [1994] along with the body size.

The somatic measurements were performed using an anthropometer, spreading caliper, sliding caliper, tape, and balance as appropriate [Hamada

**TABLE III. Locality of the Subject Populations and Number of Animals Used in the Somatometric and Pelage Color Measurements**

| | Somatometry | | Pelage color | |
|---|---|---|---|---|
| Locality | F | M | F | M |
| *M. fascicularis fascicularis (Mff)* | | | | |
| Indochina | | | | |
| WHM | 19 | 18 | 22 | 30 |
| WKT | 29 | 12 | 35 | 29 |
| Sunda | | | | |
| KN/KTK | 12 | 3 | 24 | 13 |
| WSK | 6 | 5 | 23 | 22 |
| *Mff* × *Mfa* hybrid | | | | |
| WKC | 7 | 10 | 9 | 23 |
| *M. fascicularis aurea (Mfa)* | | | | |
| BNT | 4 | 5 | 5 | 9 |
| WPN | 1 | 1 | 2 | 4 |
| MFRC | 8 | 4 | 9 | 8 |
| Total | 86 | 58 | 129 | 138 |

Code for each location refers to Table I.
F, female; M, male.

**TABLE IV. Average Proportions of Seven Body Parameters (SD, n) in the Indochinese and Sundaic *Macaca fascicularis fascicularis* (*Mff*), *M. fascicularis aurea* (*Mfa*), and Their Hybrid (*Mff* × *Mfa*)**

| | Relative tail length | Fore/lower limb length | Hand width/length | Relative third finger length | Head width/length | Relative facial length | Facial width/length |
|---|---|---|---|---|---|---|---|
| Indochinese *Mff* | 114.4 (7.6, 66) | 93.4 (2.8, 66) | 33.3 (1.4, 66) | 50.2 (2.0, 66) | 82.3 (4.3, 66) | 55.2 (5.8, 66) | 122.8 (6.7, 66) |
| Sundaic *Mff* | 129.1 (6.7, 26) | 94.7 (3.6, 26) | 32.8 (2.5, 26) | 51.1 (1.8, 22) | 83.6 (2.8, 26) | 52.7 (4.9, 22) | 129.5 (6.8, 22) |
| Hybrid[a] | 111.1 (9.0, 17) | 94.6 (2.7, 17) | 32.2 (1.7, 17) | 50.3 (1.9, 17) | 87.2 (3.7, 17) | 56.5 (5.2, 17) | 126.1 (6.1, 17) |
| *Mfa* | 108.3 (6.8, 21) | 94.2 (2.9, 21) | 32.2 (2.1, 21) | 48.8 (1.3, 21) | 83.5 (4.6, 21) | 57.8 (5.8, 21) | 124.8 (5.9, 21) |

[a]The only hybrid population which could be captured and measured the body and head proportion was at Wat Khao Chong Krachok (WKC).

et al., 2005]. Pelage color was measured at the following 10 sites on the right side of the body [Hamada et al., 2005, 2006]: crown (vertex of the head), face, back (interscapular), upper arm, forearm, hand, waist (suprailiac part of the trunk), thigh, leg, and foot. With regard to the sites on the extremities, the color was measured in the middle of the lateral or dorsal aspects of each site. For the hand and foot, the color was measured at the middle of the third metacarpal and metatarsal, respectively. The pelage color was measured using an electronic reflectometer (Color Analyzer$^{TR}$; CR-200, Minolta Co., Ltd.) as previously reported [Hamada et al., 2006, 2008], which illuminates a circular, 8-mm diameter area with standardized flashing light (D65) and measures the reflected light. The $L^*a^*b^*$ color system was used [Hamada et al., 1988], where $L^*$ stands for lightness (dark [0] to light [100]), $a^*$ for the hue of green (−60) to red (+60), and $b^*$ for the hue of blue (−60) to yellow (+60).

The morphological data are expressed as the mean ± 1 standard deviation (1 SD). The significance of differences between means was examined by *t*-test with Excel (Microsoft Co., Ltd.). Linear canonical discriminant analysis was applied on the two subspecies of *M. fascicularis* and the sub-specific hybrids using R [R Development Core Team, 2008]. We determined parameters participating in discrimination and assignment of subjects into Indochinese *Mff*, Sundaic *Mff*, or *Mfa* using a predict command which based on F-statistics. Significance was accepted at the $P < 0.05$ level.

## RESULTS

### Distribution of *Mfa* and Stone Tool Usage

Based on the survey of *Mfa* and specimens collected for this study, eight groups were discovered. The northernmost population was at Bayin Nyi Temple (BNT; 16° 58′ N, 97° 29′ E) and the southernmost was at Piak Nam Yai Island (PNY; 9° 35′ N, 98° 28′ E) (Fig. 1 and Table I). Based on the morphological characters of the crest hair pattern on the cheek and the pelage and face color, the population at Banmaisomboon School (BMS; 10° 51′ N, 99° 13′ E) was identified as *Mfa*. By observing their natural foraging behavior for 10 min to several hours, except for the BNT and PNY populations that were followed for several months, four out of the eight populations of *Mfa* were observed to use stone tools to access encased foods. The habitat types for all four groups were generally islands with rock oysters or other marine invertebrates, substrate stones, and tool stones, whereas the remaining four populations lived on the mainland (mountain for BNT and BMS, mangrove forest for MFRC, and unknown for ZDK). Interestingly, we also found one morphological hybrid population (KRI) using stone tools and their habitat was also similar to those of the *Mfa* tool users.

### Morphological Differences Between *Mff* and *Mfa*

As descriptively reported by Fooden [1995] and San & Hamada [2011] that *Mff* and *Mfa* showed different cheek and head crest hair patterns, having transzygomatic cheek hair with a head crest for *Mff* and infrazygomatic cheek hair without a head crest hair for *Mfa*, however, some groups or individual monkeys in the *Mff* groups had no head crest, but *Mfa* was never observed with a head crest in the present study (Fig. 1). Although no statistically significant difference in the pelage color between males and females was observed, the data of the two sexes were combined. Compared to the pelage color of *Mff*, *Mfa* was statistically significantly darker than both the Indochinese and Sundaic *Mff* in those 10 areas measured ($P < 0.05$; Fig. 3a). No statistically significant differences in $a^*$ (green-red hue) and $b^*$ (blue to yellow hue) were found (Fig. 3b and c), except the color at the crown, face and back were statistically significantly less yellow than those of both forms of *Mff* ($P < 0.01$; Fig. 3c). Based on the observed morphological appearances, hybrids could only be distinguished by the mixed morphological characters of asymmetric cheek hair patterns, where both patterns were observed in either side of cheek or the monkey has transzygomatic pattern of *Mff* on one

side and infrazygomatic pattern of *Mfa* on another side of cheek (KRI, SRY, and WKC), or infrazygomatic cheek hair pattern with head crest (some SRI monkeys) (Fig. 1). The pelage and face colors observed seemed to follow the geographical regions or Gloger's rule and could be separated into the Indochinese and Sundaic groups of hybrids. The Sundaic group (SRI) was slightly darker and the Indochinese groups (KRI, SRY, and WKC) were slightly lighter and similar to those of *Mff* (Fig. 1).

For the 38 somatic dimensions measured, no statistically significant differences were observed in general between *Mff* and *Mfa*, except that the Sundaic *Mff* subspecies had longer tails than those of the *Mfa* ($P < 0.05$; Table SI). Comparison of the seven proportions (ratio and indices) of morphometric dimensions, especially the body extremities that are likely to be associated with stone tool use, among the Indochinese *Mff* (WHM and WKT), Sundaic *Mff* (WSK and KN/KTK), *Mfa* (WPN, MFRC, and BNT), and morphological hybrids (*Mff* × *Mfa*; WKC) was performed (Table IV). The relative tail length showed the greatest differences among the populations, where the Sundaic *Mff* had the statistically significantly longest relative tail length ($P < 0.05$) followed by the Indochinese *Mff* and *Mfa*. Subspecies hybrids showed an intermediate relative tail length between that of the Indochinese *Mff* and *Mfa*. There were no statistically significant differences in the fore/lower limb length ratio among the different *M. fascicularis* populations studied. However, *Mfa* had a more slender hand (hand width/length ratio), relatively shorter third finger and relatively longer face, whereas the Sundaic *Mff* had the opposite trends, although the magnitude of these differences was rather small and not statistically significantly

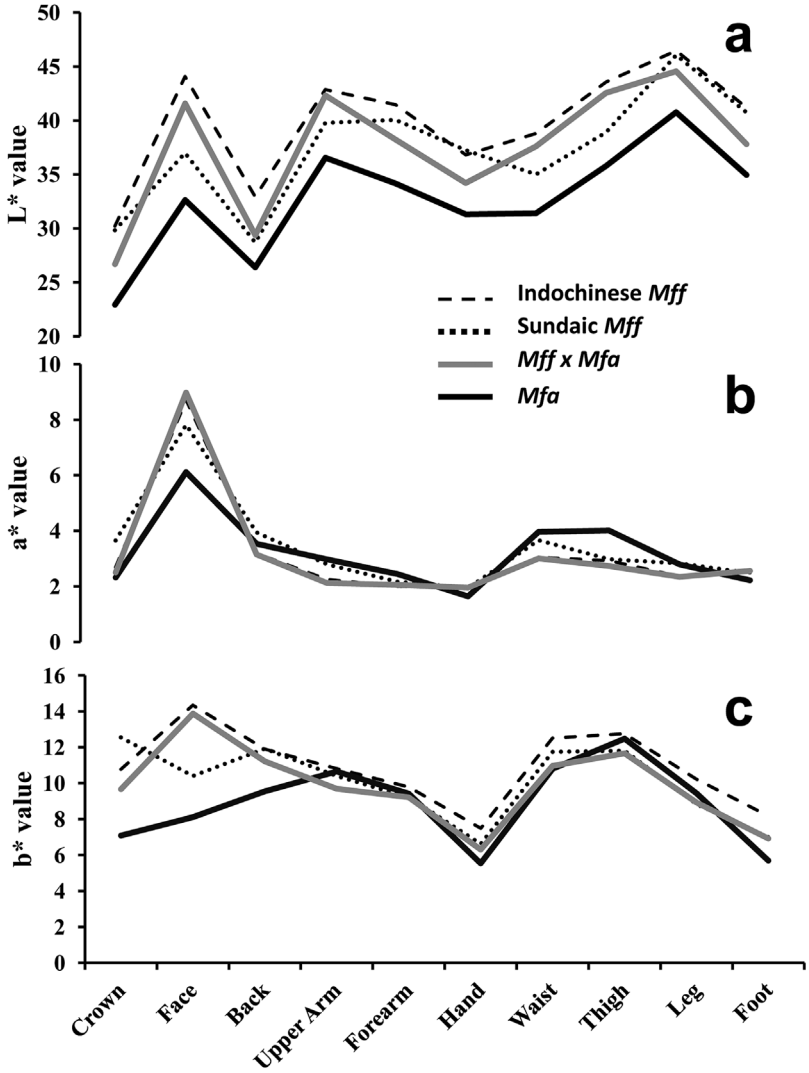


Fig. 3. Pelage color for the (a) lightness (dark [0] to light [100], L*), (b) hue of green (−60) to red (+60) (a*), and (c) hue of blue (−60) to yellow (+60) (b*) in the Indochinese and Sundaic *Macaca fascicularis fascicularis* (*Mff*), *M. fascicularis aurea* (*Mfa*), and hybrids between *Mff* and *Mfa*.

**TABLE V. Coefficients of the Linear Canonical Discrimination Function With Normalized Variables**

| | LD1 | LD2 |
|---|---|---|
| Relative tail length | 1.2440 | 0.0190 |
| Fore/lower limb length | 0.0797 | 0.2905 |
| Hand width/length | 0.1518 | −0.6585 |
| Relative third finger length | 0.4350 | −0.4048 |
| Head width/length | 0.1876 | −0.0782 |
| Relative facial length | −0.2207 | 0.4523 |
| Facial width/length | 0.3016 | 0.7148 |

different. The Indochinese *Mff* were mostly intermediate in proportion between those of the Sundaic *Mff* and *Mfa*, except for their wider hand and narrower face (facial width/length ratio).

Linear canonical discriminant analysis was applied on these seven proportions of somatic dimensions (Table V), where the relative tail length was the most statistically significant variable on LD1 followed by the relative third finger length. For LD2, the facial width/length (positively) and hand width/length (negatively) ratio were the major variables. The LD2 was plotted against the LD1 score (Fig. 4), where the Sundaic *Mff* were located on the right (positive in LD1) side of the plot, and the Indochinese *Mff* and *Mfa* were on the left (negative in LD1). *Mfa* are separated from the Sundaic *Mff* by their smaller LD1 and slightly greater LD2 scores. There are rather wide overlaps between the Indochinese *Mff* and *Mfa*, and between the Sundaic and Indochinese *Mff*. Classification by linear discrimination is listed in Table VI, where identifications were matched for 59, 16, and 13 individuals of the Indochinese *Mff*, Sundaic *Mff*, and *Mfa* and mismatched for 10.6%, 27.3%, and 43.5%, respectively. Interestingly, all of the mismatched identifications of *Mfa* were identified as Indochinese *Mff*, which was in accord with the overlap between these two groups of monkeys in the plot of the LD2 versus LD1 scores. Using the discrimination function (coefficients and constants), 3/17 sub-specific hybrids were grouped with *Mfa* and the remaining 14/17 grouped with the Indochinese *Mff*.

## Genetic Divergence of *Mfa* and *Mff*

Totally 55 and 39 monkeys were sequenced for mtDNA and Y-chromosome (Accession number: LC093173-LC093227 for mtDNA, LC093268-LC093306 for SRY gene, and LC093307-LC093345 for TSPY gene), respectively, and because four sequences of SRY and TSPY gene were downloaded from Genbank, in total of 43 sequences of Y-chromosome were analyzed. From the mtDNA sequence data, 155 bp were variable sites (22.9%) of which 142 sites were parsimony informative characters (21%), including 123 sites of two variants (18%), 10 sites of three variants (1.5%), and two sites of four variants (0.3%), and 9 sites were indels (5.8%). The numbers of transition and transversion were 127 and 7, respectively, and its ratio was 18.1:1. The variable sites in Y-chromosome (SRY and TSPY), however, were much fewer in comparison with that of the mtDNA, only 21 variable sites were found and 18 were informative parsimony (0.7%). Two indels were found of which they are a single-base indel in TSPY and a three-base indel in SRY. The numbers of transition and transversion of the Y-chromosome genes were 13 and 6, respectively, and its ratio was 2.17:1.

Based on the NJ phylogenetic analysis of 677 bp of mtDNA sequence, the monkeys were divided into

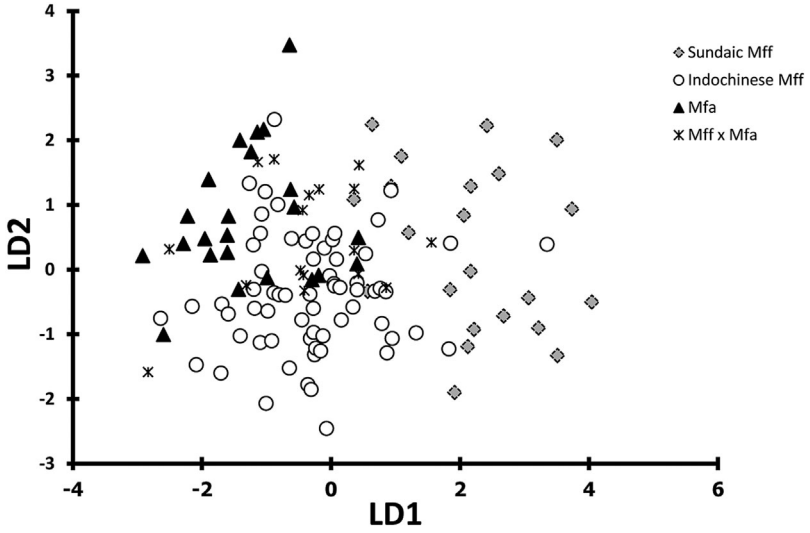


Fig. 4. Linear discrimination 1 (LD1) versus 2 (LD2) scores. Indochinese (white circle) and Sundaic (gray diamond) *Macaca fascicularis fascicularis* (*Mff*), *M. fascicularis aurea* (*Mfa*; black triangle), and hybrids between *Mff* and *Mfa* (asterisk) at WKC.

**TABLE VI. Comparison of the Identification of Indochinese and Sundaic *Macaca fascicularis fascicularis* (*Mff*) and *M. fascicularis aurea* (*Mfa*) Between the Canonical Discrimination and Morphological Appearances**

| | Canonical discrimination | | | | |
|---|---|---|---|---|---|
| Morphological identification | Indochinese *Mff* | Sundaic *Mff* | *Mfa* | Total | % Mismatch |
| Indochinese *Mff* | 59 | 3 | 4 | 66 | 10.6 |
| Sundaic *Mff* | 6 | 16 | 0 | 22 | 27.3 |
| *Mfa* | 10 | 0 | 13 | 23 | 43.5 |

the two major clades of *Mfa* and *Mm*/*Mff* (Fig. 5). Within the *Mfa* clade, the population of mainland Myanmar (BNT) was separate from the populations at the Mergui Archipelago (LPI, ZDK, and JLI) and those that originated from the Thailand Andaman seacoast (PNY and MFRC). The WPN population living on the island between Myanmar and Thailand either clustered with the Mergui populations (WPN1297) or formed a separate subclade with the Thailand Andaman seacoast populations (WPN1295 and 1298). Within the *Mm*/*Mff* clade, *Mm* was separate and *Mff* was divided into the Indochinese (WHM, KN, and WKT) and Sundaic (KN/KTK and WSK) groups. All the morphological hybrids (SRY, KRI, WKC, and SRI) clustered with the *Mff* clade. Interestingly, the BMS population, which was identified as *Mfa* by their morphological appearance, was in contrast placed within the Indochinese *Mff* clade.

The NJ analysis of the combined 686 and 2,040 bp SRY and TSPY sequences revealed that the monkeys were divided into the three major clades of *Mm*/Indochinese *Mff*, Sundaic *Mff*, and *Mfa*, respectively (Fig. 6). The Sundaic *Mff* clade consisted of the Java monkeys, peninsular Malaysia (Selangor and Johor), and Sundaic Thailand (KN/KTK and WSK). The *Mfa* clade was divided into three subclades of (i) monkeys from mainland Myanmar (BNT), (ii) the population living between Myanmar and Thailand (WPN), and (iii) island monkeys from the Mergui Archipelago (LPI and ZDK), Thailand Andaman seacoast (PNY) and mainland mangrove forest of Thailand (MFRC). Interestingly, the morphologically identified *Mfa* monkeys with the mtDNA haplotype of *Mff* (BMS) were grouped with the WPN population. The *Mm*/Indochinese *Mff* clade had only one haplotype. Three populations of mixed morphological characters identified as subspecies hybrids (KRI, SRY, and WKC) had Y-chromosome gene sequences grouped with either the *Mm*/Indochinese *Mff* or *Mfa* clades. Unfortunately, we could not collect the fecal specimens of hybrid males from the SRI population that lived close to the southernmost group of *Mfa* in the Sundaic region of Thailand. Thus, whether the SRI population grouped with the Sundaic *Mff* or *Mfa* is still unresolved.

## DISCUSSION

Based on our survey in Thailand combined with the published reports on the distribution of *Mfa* in Myanmar and Bangladesh, the geographic range of *Mfa* can be described as extending from the northernmost populations at the coastal forest belt along the Naf River at Keruntoli (20° 54′ N, 92° 16′ E), near the Teknaf Port in Bangladesh [Kabir & Ahsan, 2012], and then eastward to central and southern Myanmar in Rakhine, Ayeyarwady Delta, Bago Yoma, and Tanintharyi biogeographic regions [San & Hamada, 2011], and then southward along the Andaman seacoast to southwestern Thailand at PNY Island, Ranong Province (9° 35′ N, 98° 28′ E, this study). Note that PNY is the place where stone tool use behavior in *M. fascicularis* was first discovered in Thailand [Malaivijitnond et al., 2007] after the 120-year-old report of Carpenter [1887] in the Mergui Archipelago. Indeed, the PNY population was found in 2005, the year after the Tsunami impact on the Andaman coast of Thailand. Thereafter, as reported herein, searching for tool use populations along the Andaman seacoast and Mergui Archipelago has revealed three more locations of tool using macaques (WPN, LPI, and JLI). Although the WPN tool users were first recognized in 2007 [Malaivijitnond et al., 2007], the information was acquired from interviews of the local people, and the oyster cracking behavior using stone tools was not confirmed until this report. Indeed, another five populations of *Mfa* in Lampi National Park, Mergui Archipelago were also observed to use tools to extract oysters, but we were unable to either take photos or collect fecal samples as the monkeys were not habituated to humans and fled away quickly when they saw us.

From more than 100 populations of *Mff* surveyed in Thailand to date [Malaivijitnond & Hamada, 2008; Malaivijitnond et al., 2011], tool use has not been observed. In addition, no report on tool use in 19 other macaque species has been found [Groves, 2001]. The genetics might play an important role in the control of tool use behavior in macaques. Some support for a genetic role comes from the fact that one subspecies hybrid population (KRI), with respect to their morphological appearance and mtDNA and

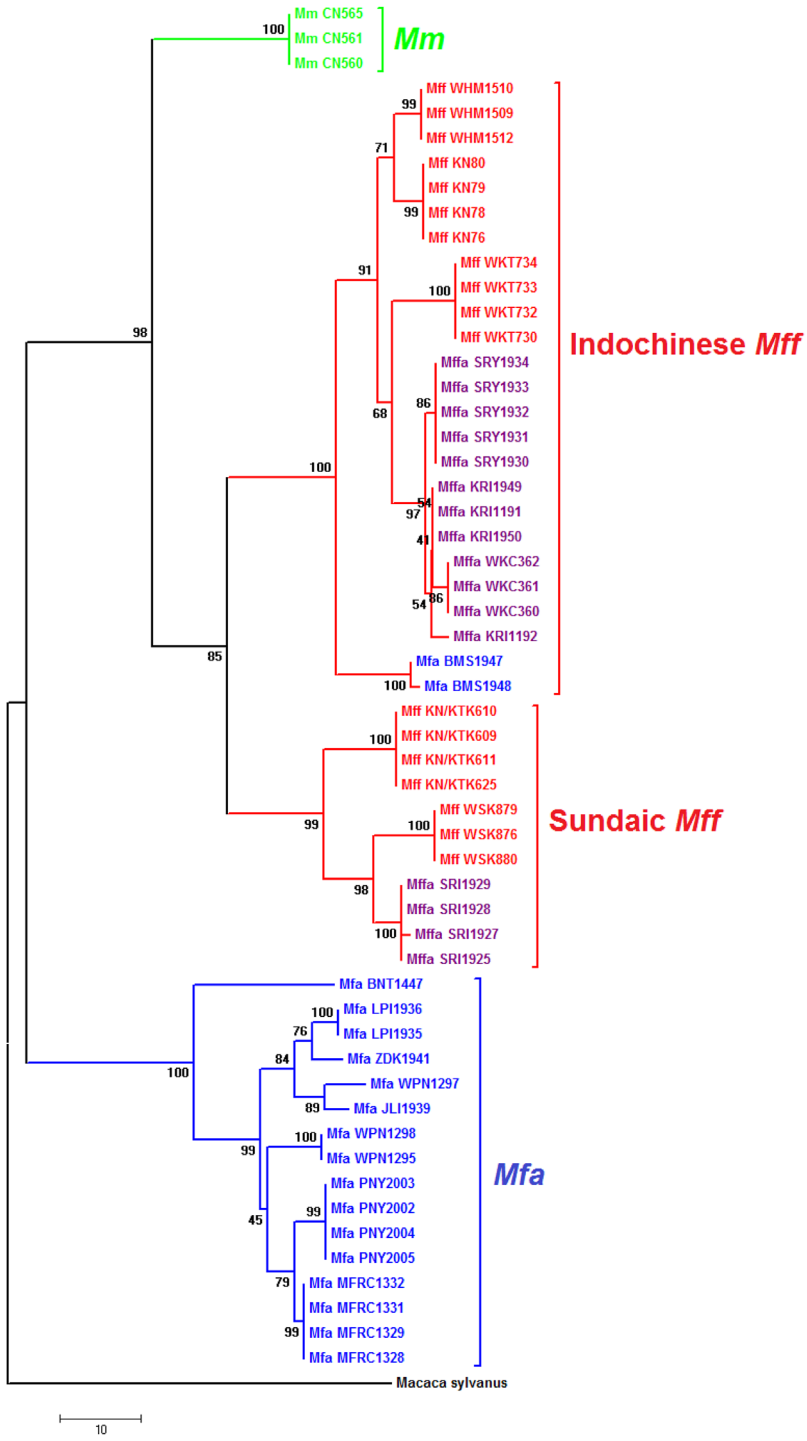


Fig. 5. NJ-based phylogenetic tree based on 677 bp of mtDNA. *Mm*, *Mff*, *Mfa*, and *Mffa* stand for *M. mulatta*, *M. fascicularis fascicularis*, *M. fascicularis aurea*, and hybrid between *M. fascicularis fascicularis* and *M. fascicularis aurea*, respectively. Green, red, blue, and purple letters indicate *M. mulatta*, *M. fascicularis fascicularis*, *M. fascicularis aurea*, and hybrid between *M. fascicularis fascicularis* and *M. fascicularis aurea*, respectively.

Y-chromosome gene analyses, was observed using stone tools. However, all the tool using macaques lived in broadly similar habitat types that consisted of encased foods, stone tools, and anvil or substrate stones. Thus, the habitat conditions could likely promote tool use behavior (necessity for acquiring food and the local availability of suitable tools) to emerge and then the behavior could propagate within and between other nearby populations. Accordingly, the natural environment is likely to

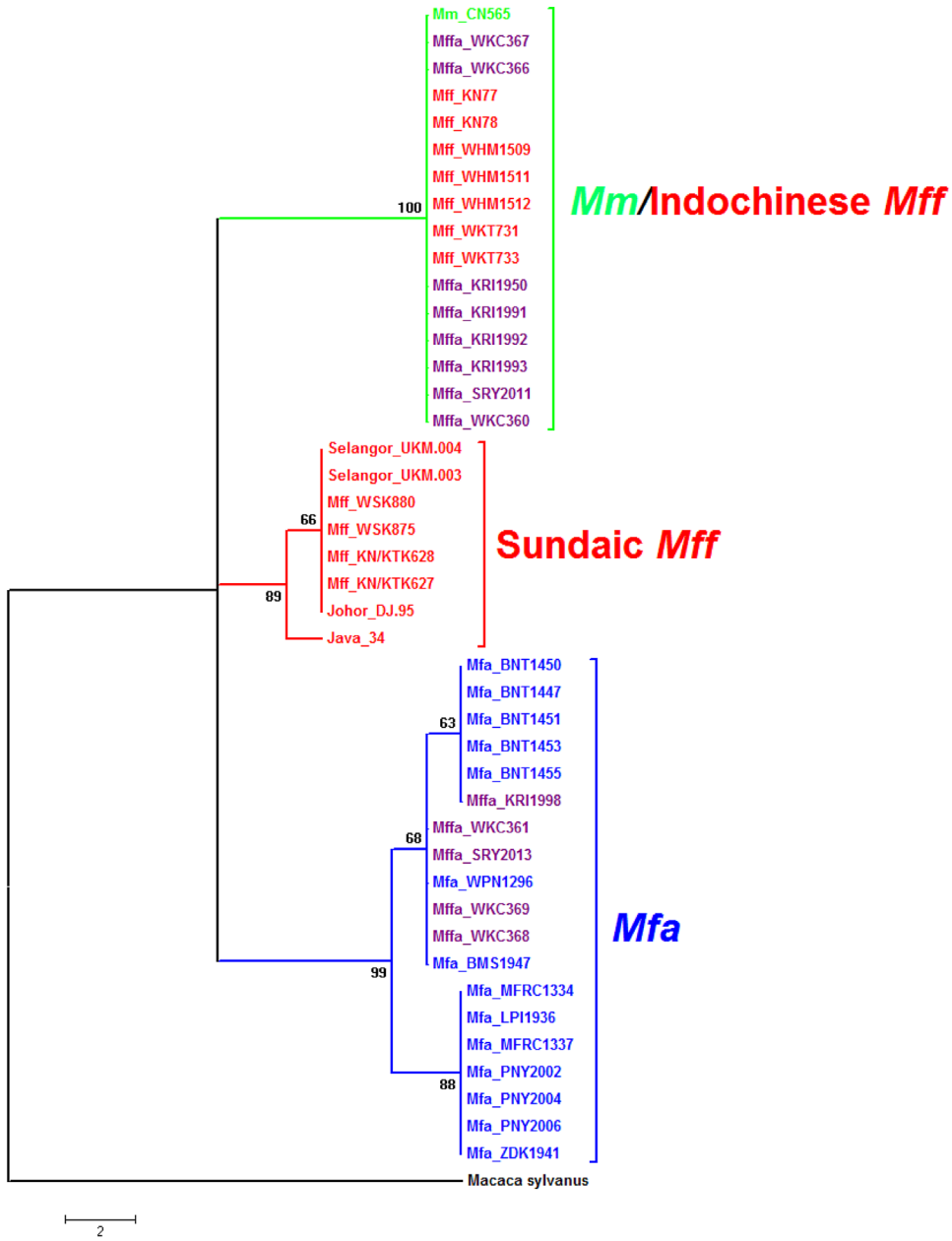


Fig. 6. NJ-based phylogenetic tree based on the 2,726 bp of the combined SRY and TSPY gene sequences. *Mm*, *Mff*, *Mfa*, and *Mffa* stand for *M. mulatta*, *M. fascicularis fascicularis*, *M. fascicularis aurea*, and hybrid between *M. fascicularis fascicularis* and *M. fascicularis aurea*, respectively. Green, red, blue, and purple letters indicate *M. mulatta*, *M. fascicularis fascicularis*, *M. fascicularis aurea* and hybrid between *M. fascicularis fascicularis* and *M. fascicularis aurea*, respectively.

be one of the driving forces for them to learn to use tools. This is also true for the isolated islands along the Mergui Archipelago and Andaman seacoast.

Regarding the morphological characteristics, *Mfa* were discriminated from *Mff* mainly by cheek hair crest, vertex hair crest, and pelage color. Although *Mfa* has partially specific body proportion than *Mff*, the classification cannot be made by such body proportions. *Mfa* has similar body proportion with Indochinese *Mff* than with Sundaic *Mff*, although the reason of similarity is not given, adaptation to the higher latitude (climate) or gene flow might be one of the reasons. Apart from the genetics and environmental force, the association of tool use in *Mfa* with their morphology was evaluated for 38 somatometric dimensions and seven somatic proportions. Except for the relative tail length and face and pelage color, no other statistically significant differences between *Mff* and *Mfa*, especially at the extremities that could be related to tool manipulation, were observed. By linear canonical discriminant analysis, *Mfa* was found to markedly differ from

Sundaic *Mff*, but was close to the Indochinese *Mff*. The significant contributing factors were the relative tail length, hand width/length ratio, relative third finger length, and relative facial length.

The mtDNA and Y-chromosome gene sequence analyses in this study supports previous studies that reported that Indochinese *M. fascicularis* had a genetic admixture of *Mm* by male *Mm* introgression [Bonhomme et al., 2009; Kanthaswamy et al., 2008; Stevison & Kohn, 2009; Tosi et al., 2002; Yan et al., 2011]. However, in this study using the SRY and TSPY gene sequences, the genetic admixture of *Mm* was only detected in the Indochinese *Mff* living in Thailand (WHM, KN, and WKY populations), but not in the Indochinese *Mfa* living in Myanmar (BNT population). Why hybrids between *Mm* and *Mfa* were not detected needs to be investigated further. Previous studies have proposed that hybrids occurred beyond the hybrid zone of 15–20 °N and that the Isthmus of Kra (10° 15′ N, 99° 30′ E) is the zoogeographical barrier in relation to changes in the sea level [Hamada et al., 2008; Kanthaswamy et al., 2010; Osada et al., 2010; Satkoski Trask et al., 2013; Tosi et al., 2000, 2002]. However, verification on this point has not been ascertained, and no macaque populations in these areas have been included for DNA analyses. This might be because it is difficult to acquire the information of free ranging *M. fascicularis* in these areas and to access those populations due to the political problem in southern Thailand. In this mtDNA-based analysis, the *Mff* inhabiting 16° 30′–13° 02′ N (WHM, KN, and WKT) and the morphological subspecies hybrids inhabiting 12° 14′–11° 48′ N (KRI, SRY, and WKC) were grouped together and separated from the Chinese *Mm* (unknown origin) and Sundaic *Mff* (8° 25′ to 7° 12′ N; WSK and KN/KTK). From the SRY and TSPY sequence analysis, all three populations of Indochinese *Mff* (WHM, KN, and WKT; 16° 30′–13° 02′ N) and the subspecies hybrids living in the Indochina region (KRI, SRY, and WKC; 12° 14′–11° 48′ N) were grouped with the Chinese *Mm*, but were separated from the peninsular (KN/KTK, WSK, Selangor, and Johor) and insular (Java) Sundaic *Mff* (8° 25′ N to approximately 7° S) populations.

The mtDNA sequence analysis indicated that the oldest *Mfa* haplotypes were from the mainland Myanmar (BNT), which supports the previous hypothesis that *Mfa* arose from a refugia population in the north of Myanmar [San & Hamada, 2011]. Considering the current geographic distribution of *Mfa* along with the mtDNA and Y-chromosome gene sequence analyses, this leads to the conclusion that *Mfa* originated in Myanmar and migrated southward along the Mergui Archipelago through the Andaman seacoast toward southwestern Thailand when the sea level was low and the Sunda shelf was exposed and the land was connected, probably in the late-Pleistocene epoch of 21,000–9,000 years ago [Sathiamurthy & Voris, 2006]. That the hybrids could be discriminated into two groups (Indochinese and Sundaic) by their pelage and face color, agrees with the two mtDNA haplotypes of Indochinese (SRY, KRI, WKC, and BMS) and Sundaic (SRI) populations of subspecies hybrids. This then denotes two possible hybridization events, namely with Indochinese or Sundaic *Mff*. In the first event, *Mfa* migrated along the Mergui Archipelago and Andaman seacoast, where male *Mfa* introgressed into Sundaic *Mff* populations, represented now by the SRI subspecies hybrids. Although *M. fascicularis* prefers low elevation habitats [Fooden, 1995], when the sea level was low, male *Mfa* living at the Andaman seacoast migrated east-northward across the low altitude area of the Dawna range [San & Hamada, 2011] to mainland Thailand and the islands on the Thai Gulf, represented now by the SRY, KRI, and WKC hybrid populations. The second hybridization event occurred recently because the males in these three populations still carried the Y-chromosome gene of either Indochinese *Mff* or *Mfa*.

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

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

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

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