Stone-tool use behavior in Burmese long-tailed macaques (stand pound) © MD Gumert

Primate (Macaque) Research and Me

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Primate Studies

วานรศึกษา

Primate (Macaque) Research and Me

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ผู้อำนวยการศูนย์วิจัยไพรเมทแห่งชาติ และ

ภาควิชาชีววิทยา คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

บทคัดย่อ

บทความนี้กล่าวถึงประวัติการทำงานวิจัยทางด้านไพรเมตของผู้เขียนที่มีมานานกว่า 30 ปี โดยเริ่มจากการให้ข้อมูลคร่าว ๆ เกี่ยวกับสัตว์ไพรเมตในประเทศไทยจำนวน 17 ชนิด ก่อนที่จะกล่าวถึงลิงมะแคค 6 ชนิดในไทยที่ประกอบด้วย ลิงกังเหนือ (*Macaca leonina*) ลิงกังใต้ (*M. nemestrina*) ลิงเสน (*M. arctoides*) ลิงอ้ายเง็ียะ (*M. assamensis*) ลิงหางยาว หรือลิงแสม (*M. fascicularis*) และลิงวอก (*M. mulatta*) โดยจะเน้นให้รายละเอียดเกี่ยวกับลิงวอกและลิงแสมเพราะเป็นมะแคคที่นิยมนำมาใช้เป็นสัตว์ทดลองทางชีวการแพทย์ จากนั้นบทความจะขมวดเข้าสู่ศูนย์วิจัยไพรเมทแห่งชาติ จุฬาลงกรณ์มหาวิทยาลัย (NPRCT-CU) และการใช้ลิงแสมสำหรับการพัฒนายาและวัคซีน โดยเฉพาะอย่างในการทดสอบวัคซีนโควิด-19

คำสำคัญ: วัคซีนโควิด-19, ลูกผสม, มะแคค, ศูนย์วิจัยไพรเมทแห่งชาติ, การใช้เครื่องมือหิน

Abstract

This article presents the historical research on non-human primates that the author has been conducting for more than 30 years. The information given started from the list of 17 species of non-human primates in Thailand, this was followed the study on 6 species of Thai macaque monkeys, the areas of expertise of the author, including northern pig-tailed macaques (*Macaca leonina*), southern pig-tailed macaques (*M. nemestrina*), stump-tailed macaques (*M. arctoides*), Assamese macaques (*M. assamensis*), long-tailed or cynomolgus or crab-eating macaques (*M. fascicularis*), and rhesus macaques (*M. mulatta*). Intensive research was conducted for the latter two macaque species that have been commonly used as animal models for biomedical research; rhesus and long-tailed macaques. The article was winded up to the National Primate Research Center of Thailand,

Chulalongkorn University (NPRCT-CU) and the use of cynomolgus macaques for drug and vaccine development especially for the COVID-19 vaccine testing.

Keywords: COVID-19 vaccine, Hybrid, Macaque, NPRCT-CU, Stone-tool use

Background

My research on non-human primates in Thailand started in 1991 when I was a Ph.D. student at Department of Biology, Faculty of Science, Chulalongkorn University. At that time, my late Thai Professor Puttipongse Varavudhi had a collaboration with my late Japanese Professor Osamu Takenka to survey and to conduct capture-and-release field work of macaques in Thailand. My first experience on monkey capturing was with Assamese macaques (*Macaca assamensis*) at Erawan Waterfall, Kanchanaburi Province. Since then, I have been working with non-human primates, mainly focusing on macaques, in Thailand and other countries in Southeast and South Asia such as Lao PDR (Laos), Vietnam, Myanmar, Indonesia, Nepal, China and India. Here, I will focus our research in Thailand.

In 1991, there were only 13 species of non-human primates listed in the Mammals of Thailand (Lekagul and McNeely 1988) including slow loris (*Nycticebus coucang*), rhesus macaque (*M. mulatta*), pig-tailed macaque (*M. nemestrina*), stump-tailed macaque (*M. arctoides*), Assamese macaque (*M. assamensis*), crab-eating macaque (*M. fascicularis*), banded langur (*Presbytis melalophos*), dusky or spectacled langur (*P. obscura*), silvered langur (*P. cristata*), Phayre's langur (*P. phayrei*), white-handed gibbon (*Hylobates lar*), pileated gibbon (*H. pileatus*), and agile gibbon (*H. agilis*). During the past 30 years, many species of non-human primates

have been discovered or re-named or re-classified and 17 species of them are listed today as follows;

1. ลิงลมซุนดาหรือลิงลมเหนือ Sunda or southern slow loris (*Nycticebus coucang*)
2. ลิงลมเบงกอลหรือลิงลมใต้ Bengal or northern slow loris (*N. bengalensis*). Formerly, slow loris in Thailand was classified as one species of; *N. coucang*, but in 2001 *N. coucang bengalensis* was raised up to be a new species of *N. bengalensis* (Groves 2001).
3. ค่างดำมลายู Banded langur (*Presbytis femoralis*)
4. ค่างอินโดจีน Germain's langur or Indochinese lutung (*Trachypithecus germaini*). Formerly, it was *T. cristatus*.
5. ค่างแว่นถิ่นเหนือ Phayre's leaf monkey (*T. phayrei*)
6. ค่างแว่นถิ่นใต้ Dusky or spectacled leaf monkey (*T. obscurus*)
7. ค่างตะนาวศรี Tenasserim lutung (*T. barbei*)
8. ลิงวอก Rhesus macaque (*Macaca mulatta*)
9. ลิงหางยาวหรือลิงแสม Long-tailed or crab-eating or cynomolgus macaque (*M. fascicularis*)
10. ลิงกังใต้ Southern pig-tailed macaque (*M. nemestrina*)
11. ลิงกังเหนือ Northern pig-tailed macaque (*M. leonina*)
12. ลิงเสน Stump-tailed macaque (*M. arctoides*)
13. ลิงอ้ายเงี๊ยะ Assamese macaque (*M. assamensis*)
14. ชะนีมงกุฎ Pileated gibbon (*Hylobates pileatus*)
15. ชะนีมือดำ Agile gibbon (*H. agilis*)
16. ชะนีมือขาว White-handed gibbon (*H. lar*)
17. เซียมัง Siamang (*Symphalangus syndactylus*)

Though there have been several species of non-human primates in Thailand, my focus is on macaque monkeys covering various subjects. Among the current 6 species of macaque monkeys living in Thailand, most intensive research was carried on long-tailed macaques including distribution and current status, morphology, behaviors, genetic diversity, heterospecific and conspecific hybridization, stone-tool use behavior and infectious diseases. During the past 30 years, the macaque research of our team has been significantly advanced by the collaboration with many institutes and universities in Thailand and aboard such as Kasetsart University, Mahidol University, and National Science and Technology Development Agency (NSTDA), Thailand; Primate Research Institute of Kyoto University, RIKEN, Tsukuba Primate Research Center, and Osaka University, Japan; Nanyang Technological University, Singapore; University of California-Davis, Arizona State University, and Dartmouth University, USA; Oxford University, UK; Max Planck Institute, and Gottingen Primate Research Center, Germany.

Research on 6 species of macaques in Thailand

• Northern (*M. leonina*) and Southern pig-tailed macaque (*M. nemestrina*)

In the past, pig-tailed macaques in Thailand was classified as only one species of *M. nemestrina*, which was classified into 2 subspecies of *M. nemestrina nemestrina* and *M. nemestrina leonina* (Fooden 1975). Based on intensive genetic (Roos et al. 2007) and morphological studies (Malaivijitnond et al. 2012), these 2 subspecies have been classified into 2 separate species. The northern pig-tailed macaque is distributed in Bangladesh, Cambodia, China, India, Laos, Myanmar, Vietnam, and Thailand; and the southern pig-tails are distributed in

southern Thailand, Malaysia, and Indonesia (Fooden 1975). The two species are parapatric at the Surat Thani-Krabi depression area (8-9°30′N), southern Thailand, where a hybrid zone was proposed (Malaivijitnond et al. 2012). Southern pig-tailed macaques were reported to be terrestrial whilst northern pig-tailed macaques were reported to be arboreal (Fooden 1975). In fact, their morphological characteristics are distinctive (Malaivijitnond et al. 2012). Northern pig-tailed macaques have a dark brown crown patch of hair that radiates from the center, and all of individuals examined had a triangle-shaped patch of white below the crown patch and above the eyes, whilst the external rim of the eyes had a red streak point up to the direction of the ears. The hair on their cheek had an approximately round shape. The muzzle was shorter than that of the southern species. The pelage showed a black streak in the middle of the back. The ischial callosity was mostly an oval shape (Figure 1). The tails of northern pig-tailed macaques were shorter and more slender compared to the southern ones, and usually arched forward over their back. Southern pig-tailed macaques had a dark brown crown patch similar to the northern pig-tailed macaques, except that it was darker. They have no triangle shape of white color below the crown patch and the red streak from the eyes. The hairs on the cheek formed an approximate balloon shape. Their pelage was black on the mid-dorsal region up to the crown patch. Their ischial callosities were butterfly shape (Figure 1). Tails of the southern pig-tailed macaques were typically arched rearwards, with the tip of the tail often directed downwards (Figure 1). The patterns of sex skin swelling and reddening, a physiological sign indicating a periovulatory period, was also different between the two species; an oval shape in northern species and a triangle shape in southern species (Figure 1).

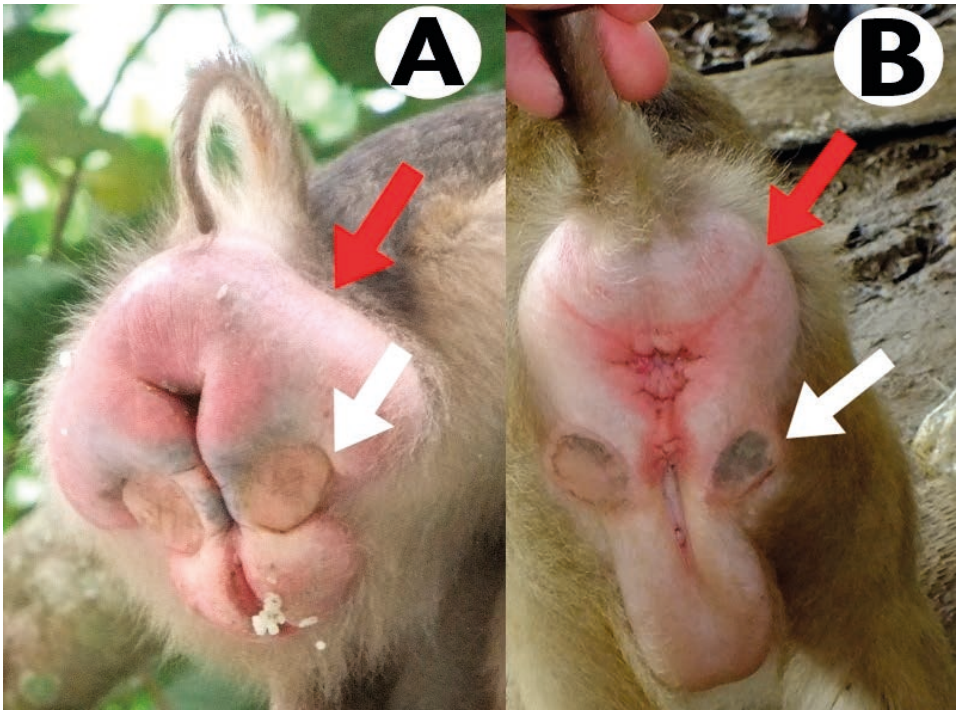

Figure 1. Ischial callosities (white arrows) and sex skin swelling pattern (red arrows) in female southern pig-tailed macaque (*Macaca nemestrina;* A) and northern pig-tailed macaque (*M. leonina;* B).

The southern pig-tailed macaques can breed year round (Fooden 1975), while the reproductive pattern of wild northern pig-tailed macaques living in Khao Yai National Park was reported as a moderately seasonal breeder (Tréboute et al. 2021). Northern pig-tailed macaques at Khao Yai National Park was also reported to perform extractive foraging behavior; targeting toxic caterpillars (Trébouet et al. 2018). Although both species have been used for coconut harvesting in Thailand, Malaysia and Indonesia, the coconut farmer prefers to use the males of northern pig-tailed macaques because of their stamina and agility (Malaivijitnond et al. 2012).

• Assamese macaques (*M. assamensis*)

Assamese macaques are recognized as two subspecies of western Assamese macaque (*M. a. pelops*) and eastern Assamese macaque (*M. a. assamensis*), and only the latter was found in Southeast Asia including Thailand (Fooden 1982). They differ in relative tail length (RTL; tail length/crown-rump length x 100) in adult males; the *M. a. pelops* has a longer tail than *M. a. assamensis*. Besides, the bridging behavior in which two adult males simultaneously lift up an infant, sucked or touched its genitalia, which facilitate the maintenance of affiliative relationships was observed only in Eastern subspecies (Ogawa et al. 2019). Eastern Assamese macaques occur in Bhutan, northeastern India, northern Myanmar, northern and western Thailand, southwest China and northern Laos and Vietnam. In Thailand, the species can be seen in Chiang Rai, Phitsanulok, Kamphaengphet, Tak, Kanchanaburi and Chaiyaphum Province (14°21′-20°19′N, 98°35′-101°39′E: Malaivijitnond et al. 2005; Sukmak et al. 2014). They are usually found at hilly areas with steep cliffs. Pelage color varies from golden brown to dark chocolate brown, and crown colored approximately like the back with parted hairs in the middle. As their RTL is comparable to that of rhesus macaque (30-50%) (Fooden 1982), they are sometimes misidentified as rhesus macaques.

Many papers on Assamese macaques in Thailand including behaviors, reproduction, infectious diseases, stress and hormone, were published by the team of Julia Ostner and Oliver Schülke. Their permanent study site is Phu Khieo Wildlife Sanctuary (Ostner et al. 2008). Using mitochondrial DNA (mtDNA) and 15 microsatellite loci of autosomal DNA, Assamese macaques in Thailand can be separated into 3 subgroups; north, west, and northeast (Sukmak et al. 2014).

Conforming with their habitat use of the hilly areas, their locomotor kinematics support the arboreal life that use the hands in a palmigrade posture and frequently flex the fingers at varying degrees (Hirasaki et al. 2019).

• Stump-tailed macaque (*M. arctoides*)

Stump-tailed macaques are distributed in eastern India from southeast of the Brahmaputra River, Bangladesh, China, Myanmar, Laos, Vietnam, Cambodia, Thailand and northernmost west Malaysia. In Thailand, their distribution is scattered in the north (Phu Khieo Wildlife Sanctuary; Fooden 1990), west (Huai Kha Khaeng and Thung Yai Naresuan Wildlife Sanctuaries, Uthai Thani; Treesucon 1988), central (Khao Krapuk Khao Taomo Non-hunting area and Kaengkrachan National Park, Petchaburi: Malaivijitnond et al. 2005; Malaivijitnond and Hamada 2005; Koyabu et al. 2008; Toyoda et al. 2017; 2018) and south of Thailand (from Prachuab Khirikhan to Trang; Boonratana 1988; Malaivijitnond et al. 2005). They are the only macaque species that newborn infants have a whitish pelage color and strong odor (Fooden 1990; Malaivijitnond and Hamada 2005; Toyoda and Malaivijitnond 2018). In both sexes of adults, the pelage color varied from reddish to light brown to dark brown to blackish, and the facial color also varied from pale pink to pink to red to dark red to blackish (Fooden 1990; Malaivjitnond and Hamada 2005). The color variation is observed within the same group, not between localities. Thus, the subspecies identification; *M. acrtoides acrtoides* and *M. a. melanota*, based on the pelage color and geography in Lekagul and McNeely (1988) is obliterated. The adults have the nearly shortest RTL (8%) among macaques (only *M. sylvanus* and some Sulawesi macaque species have shorter tails) and the forehead is nearly bald (Fooden 1990). Thus, they have

appeared to be a suitable animal model for human androgenetic alopecia study (Sundberg et al. 2001). They can breed throughout the year with a mating peak in October – November to give birth peak in May-June (Fooden 1990).

In Thailand, the study of stump-tailed macaques concentrates mainly on their behaviors such as vocalization (Toyoda et al. 2017; 2018; Claudio et al. 2013) with some reports on distribution (Boonratana 1988; Treesucon 1988; Malaivijitnond et al. 2005) and pelage color variation (Malaivijitnond and Hamada 2005; Koyabu et al. 2008). Our permanent study site of this species has been established in 2009 at Khao Krapuk Khao Taomo Non-hunting Area where various projects including genetic studies have been conducted (Toyoda et al. 2017; Toyoda and Malaivijitnond 2018; Toyoda et al. 2020). Additionally, we observed stump-tailed macaques living in sympatry and interacting (such as grooming) with long-tailed macaques at Wat Tham Khao Daeng, Nakhonsithammarat (8°14′N, 99°52′E; Malaivijitnond and Hamada 2005) and Suan Somdej Srinakharinthara, Phang-nga (8°25′N, 98°31′E; unpublished data).

• Rhesus macaque (*M. mulatta*)

Rhesus macaques are distributed in South, East, and Southeast Asia, *ca.* 15 to 36°N including Afghanistan, Pakistan, Nepal, India, Bhutan, Bangladesh, Myanmar, Thailand, Laos, Vietnam and China (Figure 2) (Fooden 2000). Their common habitat types are broadleaf evergreen forest and mixed broadleaf-needleleaf forests. They were occasionally found in needleleaf forests as well. Although most of their habitat altitudes are below 2,000 meters above sea level (asl), they were also seen as high as 3,200 meters asl in Nepal and about 4,000 meters asl in Qinghai Plateau, China (Fooden 2000). Their northern limit is determined primarily

by physiographic and climatological factors. Considering their distribution ranges, the Great Indian desert defined their north-western distribution, Himalayas and Xizang-Qinghai (Tibetan) Plateau restricted their north-central range while the transition zone between mesothermal and microthermal in China restricted their north-eastern range. The competition with neighboring species probably limits their south-eastern (competing with long-tailed macaque) and south-western (competing with bonnet macaque *M. radiata*) ranges, respectively.

Rhesus macaques were previously classified into 6 or more subspecies, however, due to inadequate information, the recognition of subspecies was obsolete and they were divided into three main groups of Eastern (China and vicinity), Western (India and vicinity) and Southern (Indochina) group (Fooden 2000; Hamada et al. 2006). The last group has smaller body size and greater in their RTL than those of Eastern (Chinese) and Western (Indian) groups (Hamada et al. 2006).

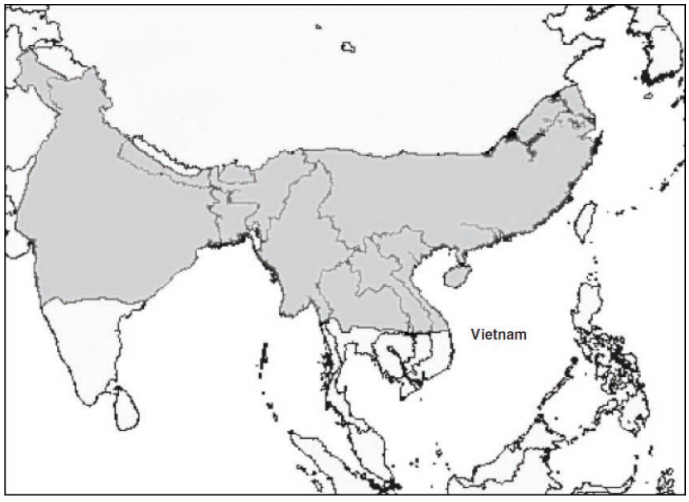

Figure 2. The distribution range of rhesus macaque is indicated by the gray color. (Adapted from Smith and McDonough, 2005)

Basically, there are two main morphological characters used for species identification in rhesus macaque. The first character is their pelage color in which the lower part of their body is more yellowish or reddish and the upper part varies from yellowish gray to golden brown to burnt orange (Fooden 2000). Another criterion is the RTL. Comparison between three rhesus groups, Chinese rhesus macaques possessed the shortest RTL (35%), followed by Indian (45%) and Indochinese (45-70%) rhesus macaques, respectively (Hamada et al. 2005; 2016). In addition to these two criteria, rhesus macaques generally have whorl hair on their cheek or sometimes form an infrazygomatic pattern where the hairs of the temporal region are smoothly directed upper-posteriorly from the posterior margin of the lip (maxilla) to the antero-lower margin of the auricular pinna (Fooden 2000; Hamada et al. 2016). Rhesus macaques give birth seasonally (Weinbauer et al. 2008). They usually have copulation and pregnancies during the fall and winter, and births during the spring and summer (Gordon 1981). Of 16 natural populations, it was reported that rhesus macaques gave births during March–August, some occurred during September–November, and none in December–February (Fooden 2000). In Hainan (18°N) and Yunnan (22°N), China, mating periods of rhesus macaques were from November–March and September–January, respectively, and their birth periods were from April–August and March–June, respectively (Fooden 1995 cf. Jiang et al. 1988). In Thailand (17°14′N), breeding season (mating-birth) of rhesus macaques was reported between November to July (Malaivijitnond and Varavudhi 2002).

• Long-tailed macaques (*M. fascicularis*)

Long-tailed macaques occupy the wide habitat ranges spreading over mainland and island in Southeast Asia between *ca.* 20° N-10° S. This macaque was commonly encountered at a low elevation in the core area, however they may extend to high altitude in some areas. Their natural habitats include seashore, mangrove forest, riverbanks, swamp forest, primary and secondary forest in the Philippines, Indonesia, Timor, Malaysia, Singapore, Cambodia, Myanmar, Thailand, Laos, Vietnam and Nicobar Islands of India (Fooden 1995). Since long-tailed macaques can adapt very well to many habitat types, they were successful to colonize in many areas beyond their natural range such as Mauritius, Island of Palau, West Papua, Tinjil Island near Java, Kabaena Island off the Sulawesi and Kowloon Hills of Hong Kong (Gumert 2011). Their northern range was defined by the interspecific competition with rhesus macaques while the southern area was restricted by the deep-sea water.

Current classification divides long-tailed macaque into 10 different subspecies; *M. fascicularis fascicularis*, *M. f. aurea*, *M. f. philippinensis*, *M. f. umbrosa*, *M. f. fusca*, *M. f. lasiae*, *M. f. atriceps*, *M. f. condorensis*, *M. f. tua* and *M. f. karimondjawae*, based on their distribution range and morphological characteristics (Fooden 1995). Among these 10 subspecies, common long-tailed macaque (*M. f. fascicularis*) spreads in the widest ranges which is found on the core area throughout mainland Southeast Asia (except Myanmar), Java, Sumatra, Borneo and many of those shallow and deep-water fringing islands (Figure 3). Due to their wide habitat ranges, common long-tailed macaque attained their name. Other two major subspecies are Burmese long-tailed macaque (*M. f. aurea*)

and Philippine long-tailed macaque (M. f. philippinensis). Burmese long-tailed macaques are distributed on the western range of the mainland from common long-tailed macaques, from Bangladesh westward to Rakhine and Tanintharyi of Myanmar and especially Mergui Archipelago, and Andaman seacoast of Thailand. Recently, it was reported that they were possibly extinct in Bangladesh (Kabir and Ahsan 2012). Since they are mainly found in Myanmar, these macaques have been named Burmese long-tailed macaques (Fooden 1995). Interestingly, Burmese long-tailed macaques possessed a unique behavior which does not exist in other subspecies of long-tailed macaques. They are well-known as stone tool-using macaques and have been reported to use stone tools to access encased foods such as nuts, shellfish and oysters (Malaivijitnond et al. 2007; Gumert et al.2009; 2011; 2013; Gumert and Malaivijitnond 2012; Falotico et al. 2017; Proffitt et al. 2018).

The last main subspecies is the Philippine long-tailed macaque. This subspecies spreads throughout the Philippines except of the southern part where common long-tailed macaques deploy (Fooden 1995). The other seven subspecies only occurred in a small number and live in some isolated islands (Figure 3).

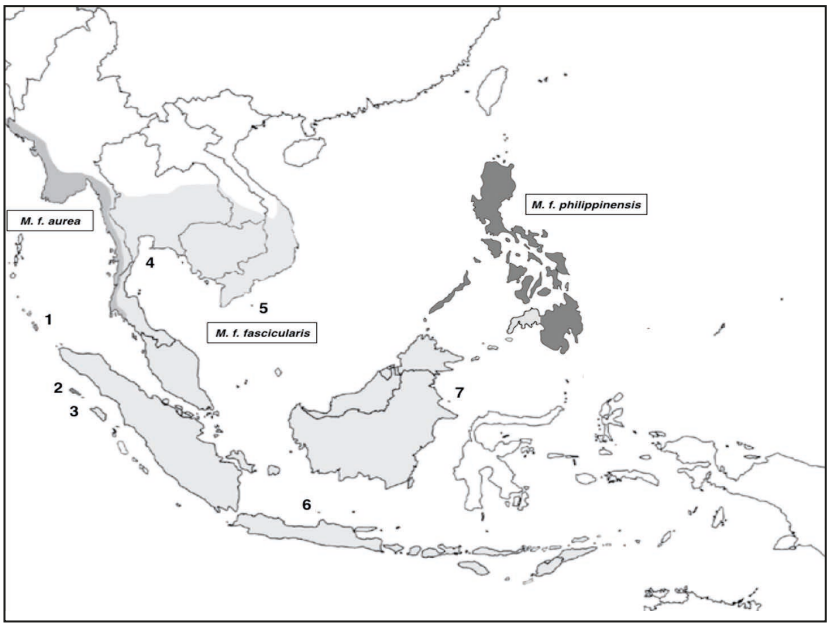

Figure 3. The distribution of long-tailed macaques. The ranges of three main subspecies which are *Macaca fascicularis aurea* (Burmese long-tailed macaque), *M. f. fascicularis* (common long-tailed macaque) and *M. f. philippinensis* (Philippine long-tailed macaque) are indicated by dark gray, light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

Among 10 subspecies, common long-tailed macaque *(M. fascicularis fascicularis*), Burmese long-tailed macaque (*M. fascicularis aurea*), and dark-crowned long-tailed macaque (*M. fascicularis atriceps*) are reported in Thailand. The dark-crowned long-tailed macaque was reported to be found only at Kram Yai Island, Chonburi province. Thus, the genetic and morphological information of this subspecies is in the status of data deficient based on the IUCN Red List of Threatened Species (2016). While common long-tailed macaques can be seen throughout Thailand, Burmese long-tailed macaques can be seen only in southwestern Thailand close to Myanmar (Malaivijitnond et al. 2007).

Long-tailed macaques are distinct from rhesus macaques, their neighboring and phylogenetically close species. Long-tailed macaques have no bipartite pattern of pelage color that is the characteristic to rhesus macaques. Their pelage colors vary from buffy to yellowish gray to golden brown to reddish brown to blackish. They have very long tails, more than 90% of RTL (Fooden 1995; Hamada et al 2016). In the case of cheek hair pattern, long-tailed macaques represent mainly transzygomatic pattern, but considerable populations of long-tailed macaques represent infrazygomatic, e.g., Burmese long-tailed macaques, and common long-tailed macaques in northern Thailand which represent rhesus macaques' character.

As for the seasonality of reproduction, following the classification of van Schaik and colleagues (van Schaik et al. 1999) and from 21 broadly distributed natural populations (Fooden 1995; 2006), long-tailed macaques are classified as moderately seasonal breeder which can give birth throughout the year but their births tended to peak during some months of the year depending on food availability. (Kavanagh and Laursen 1984; van Schaik and van Noordwijk 1985). Of 289 births, 58% occurred during the 4-month period of July–October, whilst 42% occurred during the remaining 8 months of the year (Fooden 1995; 2006). Some other studies reported that long-tailed macaques from Peninsular Malaysia (approx. 2–4°N, 101–104°E) usually gave birth at any time of the year with some peak from May to July (Kavanagh and Laursen 1984; van Schaik and Noordwijk 1985). Their inferred birth peak was during March–May in western Thailand (Fooden 1995), April–June and June–August in northern and southern Thailand, respectively (Kumpai et al. 2021). In conclusion, birth peaks of long-tailed macaques can vary locally and annually (Fooden 1995).

Biogeography of two forms of common long-tailed macaques in Thailand: Indochina and Sunda

Thailand is a hotspot for biodiversity which encompasses two different biogeographical regions; Indochina and Sunda. Indochina refers to the mainland Southeast Asia including Myanmar, Laos, Cambodia, Vietnam, and Thailand except for its southern peninsular. Sunda includes the southern peninsular of Thailand, Malaysia and Sumatra, Java, Borneo, Bali, and Lesser Sunda Islands until Timor Island. Wallace (1876) first suggested that the transition zone between these two bioregions located near the Isthmus of Kra, around 12-13°N (Figure 4). A century later, Wells (1976) studied based on bird species and found that the transition zone laid north of the Isthmus of Kra, at 10°30′N. His proposal was later supported by many other studies (Lekagul and McNeely 1988; Inger 2001). However, another transition zone, Kangar-Pattani line, which was well-known as the boundary between Indochinese-Sundaic plants among botanists and located at around 500 km further south of the Isthmus of Kra (6–6°5′N), was also proposed (Steenis 1950) (Figure 4). This suggestion was also supported by animal distribution pattern in many studies (Hughes et al. 2011; Woodruff and Turner 2009). Interestingly, Malaivijitnond et al. (2012) surveyed the distribution ranges of pig-tailed macaques and proposed the separation of these species into northern (*M. leonina*) and southern (*M. nemestrina*) populations where Surat thani-Krabi depression (~8-9°30′N), the area between Isthmus of Kra and Kangar-Pattani line, was the zoogeographical barrier. In fact, climate fluctuation in the Pleistocene epoch may have an impact on animal migration, and thus shifted the boundary between Indochinese and Sundaic northwardly or southwardly which led to the gradual change on species distribution around these regions (Tougard 2001; Hughes et al. 2011). Beside of the main zoogeographical barriers

mentioned earlier, Thailand has several biogeographical barriers such as the Tanintharyi range, the Phuket range and the Nakhon Si Thammarat mountain which could also affect animal distribution.

In Thailand, long-tailed macaques range from the lower north (16°30′N) to the southernmost (6°30′N) across the Isthmus of Kra. Thus, they were genetically clustered into two different clades (Tosi et al. 2002; Street et al. 2007; Bunlungsup et al. 2017b), and two different forms of morphological characters: Indochina and Sunda (Hamada et al. 2016) where the border between the two forms comes around Isthmus of Kra.

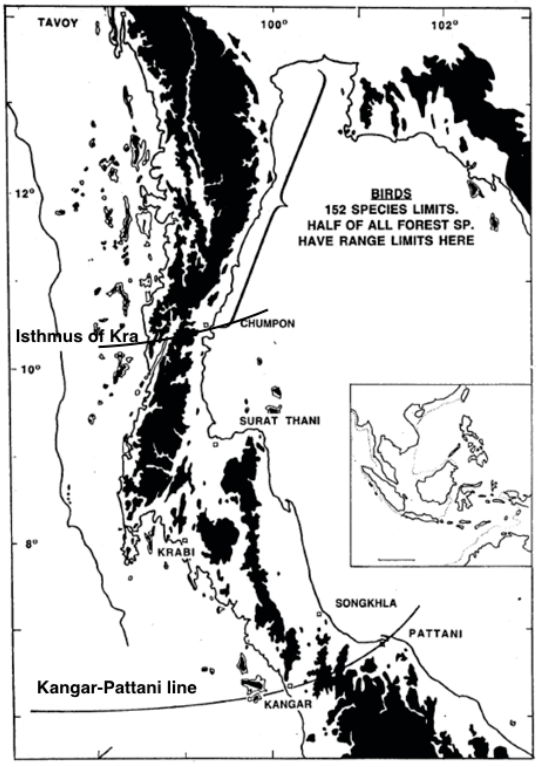

Figure 4. The map shows two main zoogeographical barriers; Isthmus of Kra and Kangar-Pattani line. (Adapted from Woodruff and Turner, 2009)

Heterospecific and conspecific hybridization in long-tailed macaques

Hybridization is considered as an important mechanism in primate evolution (Arnold and Meyer 2006). A prominent evidence is found in stump-tailed macaques where it has been shown natural hybridization can cause a formation of new taxa (Fan et al. 2018). Stump-tailed macaque is likely a result of the hybridization between the ancestors of *mulatta/fascicularis* and *sinica* species group. A more recent case was found in Sulawesi macaques where two species of *M. maura* and *M. tonkeana* were reported as hybrids in southwestern Sulawesi, Indonesia (Evans et al. 2001).

Among 23 extant macaque species, long-tailed macaques have the second largest range of distribution among non-human primate species, only exceeded by rhesus macaques (Groves 2001; Roos and Zinner 2015; Fan et al. 2017). Long-tailed macaques are distributed throughout the mainland and island of Southeast Asia between ca. 20°N to 10°S, but their northern range is considered to be restricted by the interspecific competition with rhesus macaques (Fooden 1995). Due to the change of the geography during glacial and inter-glacial periods, rhesus and long-tailed macaques had migrated southerly and northerly across their boundary and hybridized (Tosi et al. 2002). Their hybrid ranges were proposed to be at 15-20°N, encompassing four countries, that are, Myanmar, Thailand, Laos and Vietnam (Fooden 1964; Hamada et al. 2016). Since the hybrid offspring of macaques are fertile, the hybrids between these two macaque species can therefore pass their mixed genetics onto descendants (Bernstein and Gordon 1980). The hybridization scenario between these two species, including direction of the hybridization (introgression), expansion of the genetic admixture from the proposed hybrid zone and terminal location of genetic introgression, became

a focal point of interest for research over the past two-decade (Tosi et al. 2002; Kanthaswamy et al. 2008; Osada et al. 2010; Satkoski et al. 2013; Bunlungsup et al. 2017a ; Ito et al. 2020). This is because these two macaque species are the most popularly used non-human primate models in biomedical research. Yet rhesus and long-tailed macaques are different from each other in many aspects; morphology, fertility and infectious disease susceptibility, thus the hybrids between them should carry some unique characters that might share traits of their parental species or are different from either one of them.

While common long-tailed macaques can be seen almost anywhere throughout Thailand, the distribution of Burmese long-tailed macaques is restricted to southwestern Thailand (Malaivijitnond et al. 2007); and the hybridization between the two subspecies was proposed at 8°10′-12°24′N, based on the morphological characters (Fooden 1995). Overall, Thailand is the only location to study the hybridization of common long-tailed macaques with rhesus macaques (heterospecific hybridization) and with Burmese long-tailed macaques (conspecific hybridization) which makes it a very important geographic location to understand hybridization pattern in these wild macaques.

Hybridization between rhesus and long-tailed macaques in their natural habitats

According to the distribution range of rhesus and long-tailed macaques, Fooden (1995; 2000) described the parapatric zone between the two species in northern Indochina (15-20°N) covering Myanmar, Thailand, Laos and Vietnam, from west to east, respectively. Since rhesus and long-tailed macaques possess similar characteristics of glans penis as they were categorized into the same

species group by Fooden (1976), the interspecific hybridization is plausible. Besides, the hybrid offspring of macaques are fertile and can mate back either to the full-blood rhesus macaques or long-tailed macaques, leading to a broad range of genetic admixture beyond the proposed hybrid zone. Several hybridization studies based upon morphological, physiological, and genetic analysis were conducted (Tosi et al. 2002; Hamada et al. 2006; Malaivijitnond et al. 2008; Jadejaroen et al. 2015; 2016). Tosi et al. (2002) hypothesized that the hybridization between rhesus and long-tailed macaques was driven by the introgression of male rhesus macaques into long-tailed population during the Pleistocene epoch. The mtDNA phylogeny revealed the separation between rhesus and long-tailed macaques and the monophyletic pattern was found in each clade. Nevertheless, Y-chromosome phylogeny showed incongruent patterns; Indochinese long-tailed macaques were separated from the Sundaic long-tailed macaques, but grouped with rhesus macaques, resulting in paraphyly of long-tailed populations. Regarding their higher level of genetic admixture of rhesus ancestry, the morphological characters of Indochinese long-tailed macaques were similar to those of rhesus macaques, such as shorter RTL and higher body contrast, compared to the Sundaic long-tailed macaques (Hamada et al. 2008; 2016). Hamada et al. (2006) collected morphological data, for instance, body mass, body proportion, RTL, and pelage colors of rhesus macaques at Loei province, northeastern Thailand (~17°N), which is situated in the natural hybrid zone between rhesus and long-tailed macaques. Their results supported the hybridization hypothesis of which the morphological characteristics of these Thai rhesus macaques were intermediate between those of rhesus macaques in China (East) or India (West) and those of long-tailed macaques in Thailand. Jadejaroen et al. (2015) reported

the human-made hybridization between free ranging rhesus and long-tailed macaques at Khao Kheow Open Zoo, eastern Thailand that their morphological characters were intermediate between the two species; for example, 69.6-95.6% RTL and 1.33-2.01 of contrast of the pelage color (b* values). The study of human-ABO blood group in Thai rhesus and long-tailed macaques also supported the hybridization hypothesis (Malaivijitnond et al. 2008). Interestingly, Thai rhesus macaques possessed four types of ABO blood group (A, B, AB, and O) which were similar to those of Thai long-tailed macaques, but were different from their conspecific in China and India who owned only B type (Moor-Jankowski and Socha 1978; 1979).

Although many studies on the hybridization between these two species have been conducted for more than two decades, some issues, for examples; the limited area and direction of genetic introgression, were still obscured. Some studies suggested the unidirectional gene flow from male rhesus macaques to long-tailed populations were restricted to somewhere near the Isthmus of Kra (Tosi et al. 2002; Bonhomme et al. 2009; Stevison and Kohn 2009). Other studies argued against this scenario, but proposed bi-directional hybridization from rhesus to long-tailed macaques or *vice versa* (Kanthaswamy et al. 2010; Osada et al. 2010; Hamada et al. 2016). Some also proposed that rhesus gene flow was extended far beyond Indochina and southward to Sundaland, while the gene flow from long-tailed to rhesus macaques expanded northward to eastern China and India (Kanthaswamy et al. 2010; Osada et al. 2010; Bunlungsup et al. 2017a).

Hybridization scenario between common and Burmese long-tailed macaques in their natural habitats

As mentioned earlier, the common and Burmese long-tailed macaques hybridized in Thailand at Isthmus of Kra and its vicinity (12°24′N to 8°10′N) (Fooden 1995; Bunlungsup et al. 2016). These two subspecies can be generally differentiated by their lateral facial crest pattern. Common long-tailed macaques have a transzygomatic crest hair pattern, where the crest sweeps upwards from near the end of lips to the lateral margin of the crown, passing over the zygomatic bone arch locating between the eye and ear. On the other hand, Burmese long-tailed macaques have an infrazygomatic facial crest pattern, where the hairs of the temporal region are smoothly directed posteriorly from the end of lips to the anterior margin of the ear (Fooden 1995). Thus, morphological hybridization between these two subspecies was indicated by the mixed or asymmetrical facial hair patterns (Bunlungsup et al. 2016). Although the hybrid between these two subspecies were proposed, only recently we preliminary confirmed the hybrid status using mtDNA and Y-chromosome genes (Bunlungsup et al. 2016) and 868 autosomal SNP (Phadphon et al. 2021). Considering the mtDNA and Y chromosome gene sequence analyses, Burmese long-tailed macaques were proposed to originate in Myanmar and migrate southward along the Mergui Archipelago through the Andaman seacoast towards southwestern Thailand when the sea level was low and the Sunda shelf was exposed and the lands were connected, probably 21,000–9,000 years ago. Two hybridization events possibly have occurred. In the first event, male Burmese long-tailed macaques migrated along the Mergui Archipelago and Andaman seacoast, and introgressed into Sundaic common long-tailed macaque populations. This event occurred following the behavior

of long-tailed macaques that the males migrate out of the group when they reach maturity age (male dispersal), whereas females stay permanently in their native group (female philopatry) (Fooden 1995). In the second event, male Burmese long-tailed macaques living at the Andaman seacoast migrated east-northwards across the low altitude area of the Dawna range (San and Hamada 2011) to mainland Thailand and the islands on the Thai Gulf. Based on the 868 autosomal SNPs study (Phadphon et al. 2021), the hybridization between common and Burmese long-tailed macaques did not only extend far beyond the intraspecific hybrid zone that was previously proposed by Fooden (1995), but the introgression between subspecies was also occurred in discontinuous pattern across geography. One population where their morphospecies are common long-tailed macaques at Nakhonsawan province, northern Thailand (15°56′N, 99°57′E), which is approximately 500 km north of the previously proposed intersubspecific hybridization zone (8°10′-12°24′N; Fooden 1995; Bunlungsup et al. 2016) also carried the genetic admixture of Burmese long-tailed macaques (Phadphon et al. 2021).

Interestingly, the conspecific hybrid populations could use stone tool to crack open nut (Pang-nga population; Luncz et al. 2019) and mollusk (Prachuab Khirikhan population; Gumert et al. 2019; Luncz et al. 2017a; 2017b; Tan et al. 2018). Thus, genetics might substantially play an important role in the expression of tool use behavior in these hybrid macaques.

National Primate Research Center of Thailand

As mentioned above, both rhesus and long-tailed macaques are popularly used as a non-human primate models in biomedical research. This is not only because they are commercially available and commonly encountered, but their

genetic characteristics are also closely related to those of humans. Comparing with other animal models such as rats or mice, these two species of macaques are evolutionarily closer to humans. They shared a common ancestor with human dating back approximately 25 million years ago (mya) (Glazko and Nei 2003), while it was approximately 60 mya for rodents (Benton and Donoghue 2007).

The great concern of using any animal models in biomedical research is the consistency of their genetic characteristics; because using the animals with varied genetic may lead to unreliable results. For example, rhesus macaques were more susceptible to *Plasmodium knowlesi* malaria (the fifth human plasmodium) and thus had higher mortality rate than the long-tailed macaques (Schmidt et al. 1977). Since rhesus macaques exhibited severe manifestation of symptom after being infected and shared more similar post-symptoms with humans, they were suitable for malaria studies (White 2008). Thus, if researcher would use rhesus macaques with the genetic admixture of long-tailed macaques, the results might not be as expected. On the other hand, a recent publication reported that long-tailed macaques are natural hosts for five malaria parasites; *P. knowlesi*, *P. cynomolgi*, *P. coatneyi*, *P. fieldi* and *P. inui*, the first two are zoonotic and transmittable to humans (Akter et al. 2015). Recent studies have shown that long-tailed macaques throughout Southeast Asia were infected with *P. cynomolgi* and tolerant to it, while rhesus macaques are more susceptible to infections with *P. cynomolgi* and showed more severe clinical symptoms than long-tailed macaques. Similar to rhesus macaques, long-tailed macaques with varying proportion of rhesus genetics exhibited different susceptibility to malaria which was strongly associated with proportion of rhesus ancestry (Zhang et al. 2017).

As being in the tropical regions, the people of Thailand are exposed many tropical diseases. Vaccines and other medication are needed to combat those diseases. Non-human primates are required for vaccine and drug development, and they are also essential for safety and efficacy tests. With regard to these concerns and with the long-term experience on primates, as a pioneer of primate laboratory in Thailand for more than 45 years, Chulalongkorn University was designated to establish "The National Primate Research Center of Thailand (NPRCT)" in 2012. It is the first and the only primate center owned by Thais. This center aims to provide good quality research regarding health and the genetics of non-human primates and to provide research services for both in-country and aboard. The first non-human primate species which is being bred and used for research is the Thai origin long-tailed macaques. Thus, the knowledge of genetic diversity of long-tailed macaques throughout Thailand and the degree of genetic admixture with rhesus macaques in relation to their morphological characters, fertility and susceptibility to pathogen infection is important. At present, more than 600 long-tailed macaques are housed in the Center. The research facilities are composed of 6 buildings including 1 administrative building, 4 social housing breeding buildings for monkeys and 1 research building at Animal Biosafety Level-1 and 2, and we are now installing the mobile laboratory at Animal Biosafety Level-3 for efficacy test of COVID-19 vaccine.

NPRCT has been awarded the AAALAC International for Animal care and use on February 26th, 2020 and has been OECD-GLP certified on May 11th, 2021. Since the official grand opening of the Center on November 4th, 2018, our center conducted research and testing for both local and international entities. For the local institutes, the tests were mostly on safety and efficacy test of several

vaccines for tropical and emerging diseases such as Zika, Dengue and COVID-19. In the past year, our center played a crucial role in immunogenicity and safety testing for 3 types of COVID-19 vaccine: mRNA, DNA and protein-subunit. For the international entities, we mostly service on pharmacokinetics of non-communicable diseases such as diabetes mellitus, osteoporosis, and growth abnormality. Our center also focuses on two specific research themes. The first one has been focusing on genetic diversity of long-tailed and rhesus macaques in Thailand and Southeast Asian countries, and the macaque stone tool use behavior of Burmese long-tailed macaques. The second theme is a research on infectious diseases which occur in this region such as tuberculosis, malaria, B-virus, SARS-CoV-2 and microbiome. The latter focus aims to gain knowledge for drug and vaccine development to prevent or mitigate occurring symptoms. Our center has also contributed to mitigate the problem of human-macaque conflict in Thailand.

The Center has alliances with many primate centers in Asia and America, and other research institutes through MOU or MOA such as the Primate Research Institute of Kyoto University, Tsukuba Primate Research Center, and RIKEN, Japan; Southwest National Primate Research Center, California National Primate Research Center, and University of California San Diego, the United States of America; Primate Research Centre, Bogor Agricultural University, Indonesia; Beijing Institute of Genomics, Chinese Academy of Sciences, China; Chemon Inc., Korea; University of Innsbruck, Austria; Nanyang Technological University, Singapore; Advanced Medical and Dental Institute-Universiti Sains Malaysia; BioNet Asia, NSTDA and Institute of Molecular Biosciences-Mahidol University, Thailand. Regarding the ground breaking knowledge on macaque research which we have accumulated for 30 years, the NPRCT will be an important infrastructure to support and help

leveraging public health in Thailand and the world. In conclusion, basic research is essential and a very important foundation for translations into innovations.

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