ลักษณะเฉพาะทางสัณฐาน พฤติกรรมทางเพศ และสนเทศทางพันธุกรรมของลิงลูกผสมระหว่างลิงวอก *Macaca mulatta* และลิงหางยาว *M. fascicularis* ณ สวนสัตว์เปิดเขาเขียว ประเทศไทย

นางจรรยา เจตน์เจริญ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ปีการศึกษา 2558

MORPHOLOGICAL CHARACTERISTICS, SEXUAL BEHAVIORS AND GENETIC INFORMATION OF HYBRIDS BETWEEN RHESUS *Macaca mulatta* AND LONG-TAILED MACAQUES *M. fascicularis* IN KHAO KHIEOW OPEN ZOO, THAILAND

Mrs. Janya Jadejaroen

A Dissertation Submitted in Partial Fulfillment of the Requirements
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Thesis Title	MORPHOLOGICAL CHARACTERISTICS, SEXUAL BEHAVIORS AND GENETIC INFORMATION OF HYBRIDS BETWEEN RHESUS *Macaca mulatta* AND LONG-TAILED MACAQUES *M. fascicularis* IN KHAO KHIEOW OPEN ZOO, THAILAND
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จรรยา เจตน์เจริญ : ลักษณะเฉพาะทางสัณฐาน พฤติกรรมทางเพศ และสนเทศทางพันธุกรรมของลิงลูกผสมระหว่างลิงวอก *Macaca mulatta* และลิงหางยาว *M. fascicularis* ณ สวนสัตว์เปิดเขาเขียว ประเทศไทย (MORPHOLOGICAL CHARACTERISTICS, SEXUAL BEHAVIORS AND GENETIC INFORMATION OF HYBRIDS BETWEEN RHESUS *Macaca mulatta* AND LONG-TAILED MACAQUES *M. fascicularis* IN KHAO KHIEOW OPEN ZOO, THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร.สุจินดา มาลัยวิจิตรนนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.โยชิ คาวาโมโต, 111 หน้า.

ลิงวอก (rhesus macaque, *Macaca mulatta*) และลิงหางยาว (long-tailed macaque, *M. fascicularis*) จัดอยู่ในกลุ่ม *fascicularis*-species ด้วยกัน แต่อาศัยอยู่ในเขตภูมิอากาศที่แตกต่างกัน ลิงวอกอาศัยในเขตอากาศเย็น (15–35 องศาเหนือ) ในขณะที่ลิงหางยาวอาศัยในเขตร้อน (20 องศาเหนือ–10 องศาใต้) ดังนั้นลิงทั้งสองชนิดจึงมีพื้นที่อาศัยซ้อนทับกันที่บริเวณละติจูด 15–20 องศาเหนือ และมีรายงานการผสมข้ามสายพันธุ์ของลิงทั้งสองชนิดนี้ในธรรมชาติที่ยากต่อการติดตามศึกษา งานวิจัยนี้จึงได้ศึกษาลิงลูกผสมระหว่างลิงวอกและลิงหางยาว ณ สวนสัตว์เปิดเขาเขียว (ละติจูด 13 องศาเหนือ 21 ลิปดา ลองจิจูด 101 องศาตะวันออก 06 ลิปดา) ซึ่งมีรายงานว่าเกิดขึ้นจากการนำลิงวอกจำนวนหนึ่งเข้ามาปล่อยในพื้นที่อาศัยของลิงหางยาวเมื่อประมาณ 20 ปีที่แล้ว งานวิจัยนี้ติดตามศึกษาลักษณะทางสัณฐาน พันธุกรรม และพฤติกรรมทางเพศของลิงวอก ลิงหางยาว และลูกผสม การศึกษาลักษณะทางสัณฐานทำโดยการวัดขนาดจากภาพถ่าย โดยลักษณะที่ใช้ คือ ร้อยละของความยาวหางสัมพัทธ์ (%RTL) และความแตกต่างในสีเหลืองของขนบริเวณสะโพก (ด้านล่างลำตัว) และหลัง (ด้านบนลำตัว) (Cb*) ประกอบกับรูปแบบการเรียงตัวของขนที่ด้านบนสุดของหัว ที่แก้ม และการแดงของผิวหนังรอบๆ อวัยวะเพศของลิงเพศเมีย จากนั้นนำข้อมูลที่ได้มาวิเคราะห์ด้วย Multiple Correspondence Analysis (MCA) และ Agglomerative Hierarchical Cluster Analysis (AHCA) สามารถแบ่งลิงได้เป็น 5 กลุ่ม คือ ลิงที่มีลักษณะคล้ายลิงหางยาว (L_4R_0) ลิงลูกผสม (L_3R_1, L_2R_2 และ L_1R_3) และลิงที่มีลักษณะคล้ายลิงวอก (L_0R_4) ในการศึกษาลักษณะทางพันธุกรรมสามารถจำแนกลิงวอก ลิงหางยาว และลิงลูกผสม ออกจากกันได้โดยวิเคราะห์ความแตกต่างของลำดับเบส (SNP) ในยีน STAT6 ด้วยเทคนิค PCR-RFLP ทำการเพิ่มจำนวนยีน STAT6 ที่มีขนาด 745 คู่เบส นำไปตัดด้วยเอนไซม์ *Apa*I แล้วแยกชิ้นของดีเอนเอที่ได้ด้วยวิธีอะกาโรสเจลอิเล็คโทรโฟไรซิส ผลที่ได้คือแถบดีเอ็นเอจำนวน 2, 1 และ 3 แถบ ตามลักษณะจีโนไทป์ G/G ของลิงหางยาว A/A ของลิงวอก และ A/G ของลิงลูกผสม ตามลำดับ จากการวิเคราะห์ลิงจากสวนสัตว์เปิดเขาเขียวจำนวน 118 ตัวอย่าง พบจีโนไทป์ A/A, A/G และ G/G จำนวน 6 (5%), 56 (47%) และ 56 (47%) ตัวอย่าง ตามลำดับ ซึ่งสอดคล้องกับลักษณะทางสัณฐานที่วิเคราะห์ก่อนหน้านี้ นอกจากนี้ยังพบว่าเมื่อวิเคราะห์ความถี่ของอัลลีล G ซึ่งเป็นของลิงหางยาว ในระหว่างปี พ.ศ. 2549 ถึง 2557 พบว่ามีความถี่เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ จาก 0.60 เป็น 0.79 ซึ่งบ่งชี้ถึงลักษณะทางพันธุกรรมที่เบี่ยงเบนไปทางลิงหางยาวมากขึ้นในประชากรลิงกลุ่มนี้ สำหรับการศึกษาพฤติกรรมทางเพศ ได้แก่ proceptivity attractivity และ receptivity ได้คัดเลือกลิงโตเต็มวัยเพศเมียจำนวน 19 ตัว จากลิงทั้ง 5 กลุ่มที่แบ่งตามลักษณะทางสัณฐาน เก็บข้อมูลด้วยวิธี Scan sampling เป็นเวลา 6–8 วัน/เดือน ตั้งแต่ธันวาคม 2554 ถึงพฤศจิกายน 2555 พบว่าพฤติกรรมทางเพศของลิงทั้ง 5 กลุ่มมีรูปแบบที่คล้ายคลึงกันตลอดทั้งปี คือมีฤดูกาลในการผสมพันธุ์และระยะเวลาที่ให้กำเนิดลูกลิงมากที่สุดคือเดือนมีนาคมถึงพฤษภาคม โดยเฉพาะในลิงวอกที่ไม่พบการผสมพันธุ์เลยในระหว่างเดือนเมษายนและพฤษภาคม ซึ่งผลการศึกษาที่ได้นี้ไม่สอดคล้องกับรายงานก่อนหน้านี้ที่ระบุว่าลิงวอกเป็นสัตว์ที่มีฤดูกาลในการผสมพันธุ์ ในขณะที่ลิงหางยาวไม่มีฤดูกาลในการผสมพันธุ์ เมื่อพิจารณาจากลักษณะทางสัณฐาน พันธุกรรม และพฤติกรรมทางเพศของลิงที่สวนสัตว์เปิดเขาเขียว สามารถยืนยันได้ว่าลิงลูกผสมมีลักษณะต่าง ๆ ที่ก้ำกึ่งระหว่างลิงวอกและลิงหางยาว และลักษณะเหล่านี้มีความสัมพันธ์กัน

ภาควิชา ชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา สัตววิทยา ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5273895023 : MAJOR ZOOLOGY
KEYWORDS: HYBRID / RHESUS MACAQUE / LONG-TAILED MACAQUE / MORPHOLOGY / GENETICS / BEHAVIOR

JANYA JADEJAROEN: MORPHOLOGICAL CHARACTERISTICS, SEXUAL BEHAVIORS AND GENETIC INFORMATION OF HYBRIDS BETWEEN RHESUS *Macaca mulatta* AND LONG-TAILED MACAQUES *M. fascicularis* IN KHAO KHIEOW OPEN ZOO, THAILAND. ADVISOR: PROF. SUCHINDA MALAIVIJITNOND, Ph.D., CO-ADVISOR: ASSOC. PROF. YOSHI KAWAMOTO, Ph.D., 111 pp.

Rhesus (*Macaca mulatta*) and long-tailed (*M. fascicularis*) macaques are in the same *fascicularis* species group but live in the different climate zones. Rhesus macaques inhabit the cooler climate (15–35°N) while long-tailed macaques inhabit the warmer climate (20°N–10°S). The natural hybrid zone between these two species is 15–20°N, and difficult to study. This work focused on a hybrid population between these two species which was recently found in Khao Khieow Open Zoo, (KKZ; 13°21′N, 101°06′E) where a group of rhesus macaques was released into the habitat of feral long-tailed macaques about 20 years ago. Their morphological and genetic characteristics and sexual behaviors were evaluated. Photogrammetric method was used to assess %relative tail length (RTL) and contrast of the yellow pelage color (Cb*). %RTL, Cb* together with crown hair, cheek hair, and sex skin reddening were analyzed by Multiple Correspondence Analysis (MCA) and Agglomerative Hierarchical Cluster Analysis (AHCA) and categorized the KKZ macaques into 5 groups of long-tailed like (L_4R_0), hybrid (L_3R_1, L_2R_2 and L_1R_3), and rhesus-like (L_0R_4) macaques. Genetic discrimination of the two species and hybrids was done by STAT6 SNP analysis using PCR-RFLP technique. The 745 base pair fragment of STAT6 gene was digested with *Apa*I and gel electrophoresed which gave two, one and three band patterns that correspond to the G/G genotypes of long-tailed, A/A genotype of rhesus and A/G genotype of hybrid macaques, respectively. Of 118 KKZ samples, 6 (5%), 56 (47%), and 56 (47%) showed A/A, A/G, and G/G genotype, respectively, which were corresponded to their morphological characteristics. The frequency of G (long-tailed) allele in the population from the year 2006 to 2014 was significantly increased from 0.60 to 0.79, indicating a cline to long-tailed macaques. Nineteen adult females were selected from those 5 groups of KKZ macaques, and sexual behaviors including proceptivity, attractivity, and receptivity were collected by scan sampling method, 6–8 days/month from December 2011 to November 2012. All 5 monkey groups showed comparable patterns of sexual behaviors throughout the year. The patterns depicts breeding season with birth peak during March and May, especially in rhesus-like macaque group of which the receptivity was not seen during April and May. The results gained from this study are incongruent with previous reports indicating that rhesus macaques are strict seasonal breeders and long-tailed macaques are non-seasonal breeders. In regard to the morphological, genetic and behavioral studies of KKZ population, it confirms that the hybrids between rhesus and long-tailed macaques show intermediate characteristics between the two species and these characteristics are associated with each other.

Department:	Biology	Student's Signature	.....
Field of Study:	Zoology	Advisor's Signature	.....
Academic Year:	2015	Co-Advisor's Signature	.....

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CHAPTER I

GENERAL INTRODUCTION

Rhesus (*Macaca mulatta*) and long-tailed (*M. fascicularis*) macaques have been known as the two most commonly used non-human primate models for biomedical research (Tosi et al., 2002; Gibbs et al., 2007; Kanthaswamy et al., 2008; 2010; Bonhomme et al., 2009; Stevison and Kohn, 2009; Osada et al., 2010; Barr et al., 2011; Satkoski Trask et al., 2013). Rhesus macaques inhabit mainland of southern Asia (15–35°N) with cooler climate including Afghanistan eastward to India and China, and southward to Myanmar, Thailand, Laos and Vietnam (Fig.1.1) (Fooden, 2000; 2006; Malaivijitnond and Varavudhi, 2002; Hamada et al., 2005a; 2006). Long-tailed macaques inhabit Peninsular and insular Southeast Asia (20°N–10°S) with warmer climate including Bangladesh, Myanmar, Thailand, Laos, Cambodia, Vietnam and southward to Malaysia, Singapore, Brunei, Indonesia, the Philippines and Timor (Fig.1.1) (Fooden, 1971; 2006; Hamada et al., 2008; Malaivijitnond and Hamada, 2008). Rhesus and long-tailed macaques are parapatric or marginally sympatric that their inferred natural interspecific border extends approximately 2,000 km from southeastern Bangladesh eastward through southern Myanmar, northern Thailand, southern Laos and central Vietnam at about 15–20°N (Fig.1.1) (Hamada et al., 2005a; 2006; Fooden, 2006).

The hybridization between rhesus and long-tailed macaques has occurred more than 1.0 million years ago in glacial period of Pleistocene Epoch (Tosi et al., 2002; 2003; Osada et al., 2010). Up to now, there are still a limitation of knowledge on hybrid characteristics particularly in morphology and behavior. Morphologically, the two species show different appearances (Fooden, 1995; 2000; 2006; Hamada et al., 2005a; 2008; Malaivijitnond et al., 2007a). Rhesus macaques usually have relatively short tails (Hamada et al., 2005a; 2005b; 2008; Fooden, 2006). They have contrast of pelage color which is more yellowish and reddish at the lower part of their back comparing to the upper part (bipartite pattern) (Hamada et al., 2005a; 2006). Long-tailed macaques have much longer tails without bipartite pelage color pattern (Hamada et al., 2006; 2008; Fooden, 2006). The two species also show different patterns of crown hair, cheek hair

and sex skin reddening (Fooden, 1995; 2000; Engelhardt et al., 2005; Hamada et al., 2006; 2008; Malaivijitnond et al., 2007a).

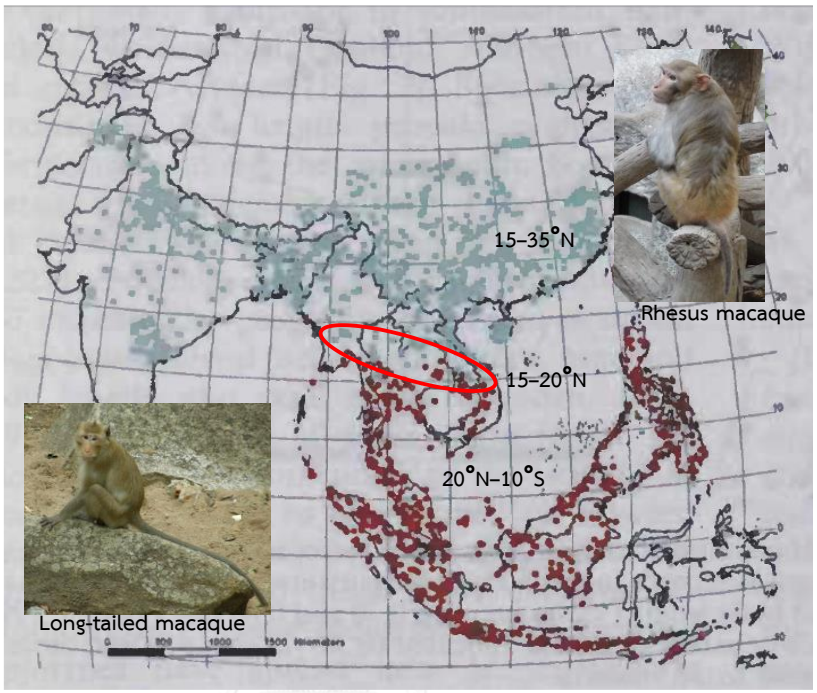

Figure 1. 1 Distribution range of rhesus (upper part) and long-tailed macaques (lower part) with their purposed hybrid zone (in circle). Map adapted from Fooden (2006).

According to their reproductive seasonality, as rhesus and long-tailed macaques have been adapted to different climate as mentioned above, the former are seasonal breeders (Fooden, 2000; 2006) while the latter are non-seasonal breeders which can copulate and give birth throughout the year (Kavanagh and Laursen, 1984; van Noordwijk and van Schaik, 1985; Fooden, 1995; 2006). Until the present time, there is a lack of report on the pattern of sexual behaviors of the hybrids between these two species.

There are some genetic data of hybrids between rhesus and long-tailed macaques which are very important for translational to medical research and evolutionary study (Fooden, 1997; Fooden and Albrecht, 1999; Fooden, 2000; Tosi et al., 2002; Hamada et al., 2006; 2008; Malaivijitnond and Hamada, 2008; Malaivijitnond et al., 2008; Bonhomme et al., 2009; Barr et al., 2011). Single nucleotide polymorphism

(SNP) of STAT6 (signal transducer and activator of transcription) gene located on the macaque chromosome 11 was found and used to discriminate both species and hybrids between them (Barr et al., 2011). However, most of the techniques used were expensive (Boyle et al., 2004; Muniesa et al., 2014) and inaccessible in the range countries of these two species.

As mentioned above that hybrids between rhesus and long-tailed macaques had occurred for a very long time ago (Tosi et al., 2002; 2003; Osada et al., 2010) however, tracing and studying those hybrids in their natural habitats could be difficult. The study on recent human-caused hybrid population is possible and will lead to more understanding of their hybridization process. In 2008, there was a report on hybrids recently occurred in Khao Khieow Open Zoo (KKZ; 13°21′N, 101°06′E), Chonburi Province (Malaivijitnond and Hamada, 2008) (Fig.1.2). This zoo was founded in 1978 and from the interview of a senior zoo staff, some rhesus macaques were released (approximately 20 years ago) in the forested area of the zoo which locates outside the distribution range of rhesus macaques. It has been reported that they have lived together with long-tailed macaques that are indigenous to that area. It is then believed that the released rhesus macaques have had possibility to copulate with long-tailed macaques because some members of that population showed mixed and intermediate morphological characteristics between the two parental species at the present time (Malaivijitnond et al., 2011).

By the preliminary survey from the end of 2009 to 2011, it was found that there were three main groups of long-tailed macaques in KKZ with overlapped home range (Fig. 1.2). Group 1, with many macaques with mixed morphological characteristics of rhesus and long-tailed macaques, was chosen as the study group. The focal group was followed and habituated during 2010 and 2011. The group size was estimated in January 2011. It is found that group 1 composed of 287 members and had relatively largest home range (Fig. 1.2). Group 2 and 3 had 98 and 115 members, respectively and both with relatively smaller home range sizes when comparing with that of group 1. During 2011, there were only 6 adult males with only a rhesus-like individual and they were with dispersal behavior. Thus, this study focused on the 60 adult females

for the study of their morphological traits of rhesus-like, long-tailed like, and mixed between the two species.

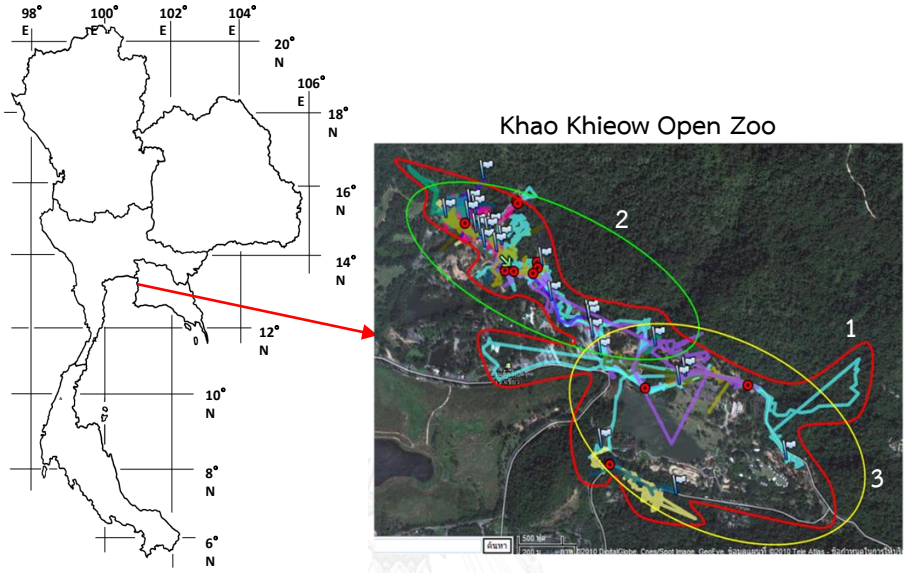

Figure 1. 2 Location of Khao Khieow Open Zoo (KKZ) in Thailand with the approximate home range, encompassing in a total of 800 ha both in the exhibition areas and natural mixed deciduous and dry evergreen forest, of the three main free-ranging groups; 1 (red line), 2 (green line) and 3 (yellow line) (Map from Google Earth accessed in January 2011).

All the adult individuals were named according to their unique morphological, personal behavioral, or other characteristics before the morphological study which was by taking their photographs and assessing the morphological traits from their archived photographs. DNA of these females including hybrids were assessed using the STAT6 SNP according to Barr et al. (2011) but by applying relatively simple and inexpensive assay for the discrimination of hybrids. Sexual behaviors were then observed for a year to see whether or not individuals with rhesus-like morphological trait with rhesus genotype and long-tailed like morphological trait with long-tailed genotype were seasonal and non-seasonal breeders, respectively, and to assess such the pattern of hybrids between the two species.

Morphological characteristics provide suitability for an organism's survival and reproductive success at its present time. Genetic composition of current organisms are the results of their evolution from the past. However, behavioral characteristics are not only derived or are genetically based from the past, but also occur together with learned behaviors in order to cope with their biotic and abiotic environment at the present time where they live.

Thus, the study of hybrids between long-tailed and rhesus macaques recently occurred in KKZ through their morphological characteristics, genetics and sexual behaviors is thus expected to be beneficial for the understanding of hybridization between these two species which occurred in the past in their natural hybrid zone. In addition, comparing of the morphological, genetic and behavioral characteristics of the hybrids between these two species has not been reported before. The comparisons will depict the hybrids in various and wider angles.

The experimental protocol in this study was approved by the Animal Ethical Committee in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University, Protocol Review No. 1323010.

Objectives

1. To study morphological characteristics of hybrids between long-tailed and rhesus macaques using photogrammetric methods
2. To investigate simple and inexpensive genetic marker for the determination of these hybrids
3. To determine whether these hybrids are seasonal or non-seasonal breeders

CHAPTER II
LITERATURE REVIEW

Rhesus macaques *Macaca mulatta*, Zimmermann, 1780 and long-tailed or cynomolgus or crab-eating macaques *M. fascicularis*, Raffles, 1821 have been known as the two most commonly used non-human primates for biomedical research (Gibbs et al., 2007). They live parapatrically or marginally sympatrically as shown in Fig. 1.1 (Fooden, 2006). As mentioned in Chapter I, rhesus macaques distribute in Afghanistan, India and China, Myanmar, Thailand, Laos and Vietnam while long-tailed macaques are in Bangladesh, Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia, Singapore, Brunei, Indonesia, the Philippines, and Timor (Fig. 1.1) (Fooden, 2000; 2006). The natural hybridizations between these two species which occurred in the past over a million years ago were reported and the proposed hybrid zone was at 15–20°N in Indochina Peninsular (Fig. 1.1) (Fooden and Albrecht, 1999; Tosi et al., 2002; Fooden, 2006; Hamada et al., 2005a; 2006; 2008; Osada et al., 2010). Although many studies on hybrids between these two species have been conducted, the vast majority of research focuses on genetics, not on morphology and behavior. It might be because the latter two approaches are difficult to conduct in free-ranging or in wild animals and usually take long time for completion. The followings are basic and scientific information of these two species.

Taxonomy of rhesus and long-tailed macaques

Rhesus and long-tailed macaques are in Order Primates and Parorder Catarhini where Catarrhines refer to both Old World monkeys and apes. Rhesus and long-tailed macaques belong to Family Cercopithecidae or Old World monkey that their nostrils face downwards (narrow-nosed) (Groves, 2001). Macaques are classified in Subfamily Cercopithecinae according to the cheek pouches and simple stomachs. Macaques belong to the genus *Macaca* (Lacépède, 1799), of which "*Macaca*" comes from a Portuguese word of "macaco" that means "monkey". This genus consisted of 19–22

species with a number of subspecies (Fooden, 1980; Groves, 2001; Anandam et al., 2013; Zinner et al., 2013).

Fooden (1980) classified all macaques into four species groups; *silenus-sylvanus, sinica*, *fascicularis* and *arctoides*. Both long-tailed and rhesus macaques belong to the same species group of *fascicularis* of which males have bluntly bilobed and narrow glans penis. However, Groves (2001) classified them, based mainly on the mitochondrial ribosomal gene (12S and 16S) analysis, into six species groups; *M. sylvanus*, *M. nemestrina*, Sulawesi, *M. fascicularis*, *M. mulatta*, and *M. sinica*. Long-tailed macaques are proposed into *M. fascicularis* group while rhesus macaques are in *M. mulatta* group. Anandam et al. (2013) and Zinner et al. (2013) classified macaques into 22 species in seven species groups; *M. sylvanus*, *M. silenus*, Sulawesi macaque, *M. sinica*, *M. arctoides*, *M. mulatta*, and *M. fascicularis*. *M. mulatta* group composed of rhesus macaques, Taiwanese macaques *M. cyclopis*, and Japanese macaques *M. fuscata*.

Zhang and Shi (1993) classified rhesus macaques into six subspecies; *M. mulatta mulatta*, *M. m. brevicaudus*, *M. m. lasiotis*, *M. m. littoralis*, *M. m. vestita*, and *M. m. tcheliensis.* Groves (2001) classified them into six subspecies; *M. m. mulatta*, *M. m.villosa*, *M. m. vestita*, *M. m. lasiota*, *M. m. sanctijohannis and M. m. brevicauda* among which *M. m. mulatta* distributed in Thailand, Myanmar, and Vietnam. Brandon-Jones et al. (2004) classified them into seven subspecies; *M. mulatta mulatta*, *M. m. lasiota*, *M. m. littoralis*, *M. m. sanctijohannis*, *M. m. siamica*, *M. m. tcheliensis* and *M. m. vestita*. Among these, Indochinese rhesus macaques (*M. mulatta siamica*) distributed in south-central Yunnan, north Thailand, and northern Vietnam. However, Fooden (2000) abandoned subspecific classification but classified them into East (Chinese and vicinity) and West (India and vicinity) groups.

Long-tailed macaques are classified into 10 subspecies; *M. f. fascicularis*, *M. f. philippinensis*, *M. f. aurea* (or *aureus*), *M. f. atriceps*, *M. f. condorensis*, *M. f. fusca* (or *fuscus*), *M. f. karimondijawae*, *M. f. lasiae, M. f. tua*, and *M. f. umbros* (or *umbrosus*) (Groves, 2001; Brandon-Jones et al., 2004; Anandam et al., 2013; Zinner et al., 2013).

The scientific name of rhesus macaques *M. mulatta* is from Spanish or Portuguese *mulatto(a)* that means “of mixed breed” (Roos and Zinner, 2015). The

complex human Rh blood group is also named after the rhesus macaques (Dean, 2005). The scientific name of long-tailed macaques, *M. fascicularis*, comes from Latin which means "a small band or stripe" (Roos and Zinner, 2015). Long-tailed macaque is named because of its longest tail among macaque species whereas cynomolgus macaque was derived from Greek word *Kynamolgoi* which means "dog milkers" (Roos and Zinner, 2015).

Geographical distribution

Among all macaques, rhesus and long-tailed macaques are the two species that the habitat encompass the largest area in Asia. Rhesus macaques distribute in subtropical area (15–35°N) with cooler climate, distributing from Afghanistan eastward to India and China, and southward to Myanmar, Thailand, Laos and Vietnam (Fooden, 2000) (Fig. 1.1). This species was previously lived up to 40°24′N, approximately 160 km northeast of Beijing, China; however, the last ones were killed in 1987 by a local hunter (Yongzu et al., 1989). Rhesus macaques are broadly classified into three groups of eastern (China and vicinity), western (India and vicinity) and southern group (Indochina) (Fooden, 2000; Hamada et al., 2006).

Long-tailed macaques distribute in tropical area (20°N–10°S) with warmer climate from Bangladesh eastward to Myanmar, Thailand, Laos, Cambodia and Vietnam, and southward to Malaysia, Singapore, Brunei, Indonesia, the Philippines and Timor (Malaivijitnond and Varavudhi, 2002; Malaivijitnond et al., 2005; Fooden, 2006). Long-tailed macaques were also introduced by human to the island of Mauritius in the Indian Ocean, east of Madagascar (Fooden, 1976) (Fig. 1.1). Largely, the typical subspecies of long-tailed macaques (*M. fascicularis fascicularis*) are divided into two groups of Indochinese and Sundaic long-tailed macaques where the Isthmus of Kra (10°28′N, 98°48′E) is the natural barrier. Indochinese long-tailed macaques lived north of Isthmus of Kra, and the Sundaic one lived south of Isthmus of Kra.

The natural interspecific boundary of these two species is about 15–20°N (Fig. 1.1) along their inferred natural interspecific border extending approximately 2,000 km from southeastern Bangladesh eastward through southern Myanmar, northern Thailand, southern Laos and central Vietnam where hybridization was recognized in

regard to morphological and genetic characteristics (Fooden and Albrecht, 1999; Fooden, 2006; Tosi et al., 2002; Hamada et al., 2005a; 2006; 2008). The rhesus and long-tailed macaques in this hybrid zone, situating the Indochina, were so-called Indochinese rhesus and long-tailed macaques, respectively.

In Thailand, rhesus macaques distribute in northeastern part from 16°27′N in Phu Khiao Wildlife Sanctuary, Chaiyaphum Province to 18°12′N in Nongkhai Province. Long-tailed macaques distribute from the northern and northeastern parts of Thailand from 17°06′N in Udonthani Province downward to 6°30′N in Yala Province, the southernmost part of the country. Thailand encompasses the distribution ranges of both Indochinese and Sundaic populations of long-tailed macaques which are separated by Isthmus of Kra (Malaivijitnond et al., 2005; Malaivijitnond and Hamada, 2008).

Morphological characteristics

Main morphological characteristics; tail length, contrast of dorsal pelage color (yellow hue) between waist and back (bipartite pattern), and patterns of crown hair, cheek hair and sex skin reddening of rhesus and long-tailed macaques are shown in Figure 2.1. In a measurement of body size, the values of head and body length (HBL) (Fooden, 1995; 2000; 2006) and crown-rump length (CRL) (Hamada et al., 2005a; 2005b; 2006; 2008; 2015) are not exactly the same. HBL is the measurement from the tip of the nose while CRL is measured from the crown, respectively, to rump of a macaque which are the measurement in mammalogy and anthropology, respectively. This study cited both HBL and CRL but compared data with CRL and % relative tail length (%RTL) calculated from CRL and tail length (Hamada et al., 2005a; 2005b; 2006; 2008; 2015). Detailed of morphological characteristics of each species are as the followings.

Rhesus macaques

Rhesus macaques have dimorphism in body size that males are approximately 44% heavier and 13% larger (in head and body length) than the females (Fooden, 2000). Adult males weigh 7.70 ± 2.33 kg (range 4.01–14.06, n = 25) while adult females weigh 5.34 ± 1.34 kg (range 3.00–9.98, n = 33). Head and body length of adult males

and females are 531.8 ± 55.2 mm (range 410–660, n = 48) and 468.8 ± 49.1 mm (range 370–580, n = 72), respectively (Fooden, 2000).

Relative tail length (%RTL; tail length/HBL x 100) of male and female rhesus macaques are 43.5± 7.9 (ranging 20.0–62.0, n = 48) and 45.0± 9.6 (range 27.8–72.1, n = 72), respectively (Fooden, 2000). Relative tail length (%RTL; tail length/CRL x 100) of Chinese, Indian (n = 17) and Thai rhesus macaques were 36.5 ± 2.85 (n = 36), 44.2 ± 4.91 (n = 17), and 53.9 ± 3.35 (n = 12), respectively (Hamada et al., 2006). %RTL of rhesus macaques is a species-specific character which is approximately < 70% (Fig. 2.1) (Hamada et al., 2015).

Pelage color includes lightness (L*), red-green hue (a*), and yellow-blue hue (b*). There is no sex different in the pelage color of rhesus macaques (Fooden, 1995; Hamada et al., 2005a; 2005b; 2006). Dorsal pelage coloration is also a key character for species identification of rhesus macaques that the fur of the lower back is conspicuously more red (a*) and yellow (b*) than that of the upper back, which is called bipartite pattern of pelage color (a* or b* value of the lower back subtracting by a* or b* value of the upper back) (Fooden, 2000; Malaivijitnond and Varavudhi, 2002; Hamada et al., 2015). The color of the upper back varies from yellowish gray to golden brown to burnt orange, and the color of the lower back varies correspondingly from golden brown to burnt orange to intense burnt orange (Fooden, 2000).

Dorsal pelage color of newborn infant of rhesus macaques is dark brown to blackish, conspicuously darker than in adults. This character is the same in newborn infants of other species in *fascicularis* group; long-tailed, Taiwanese, and Japanese macaques (Fooden, 2000).

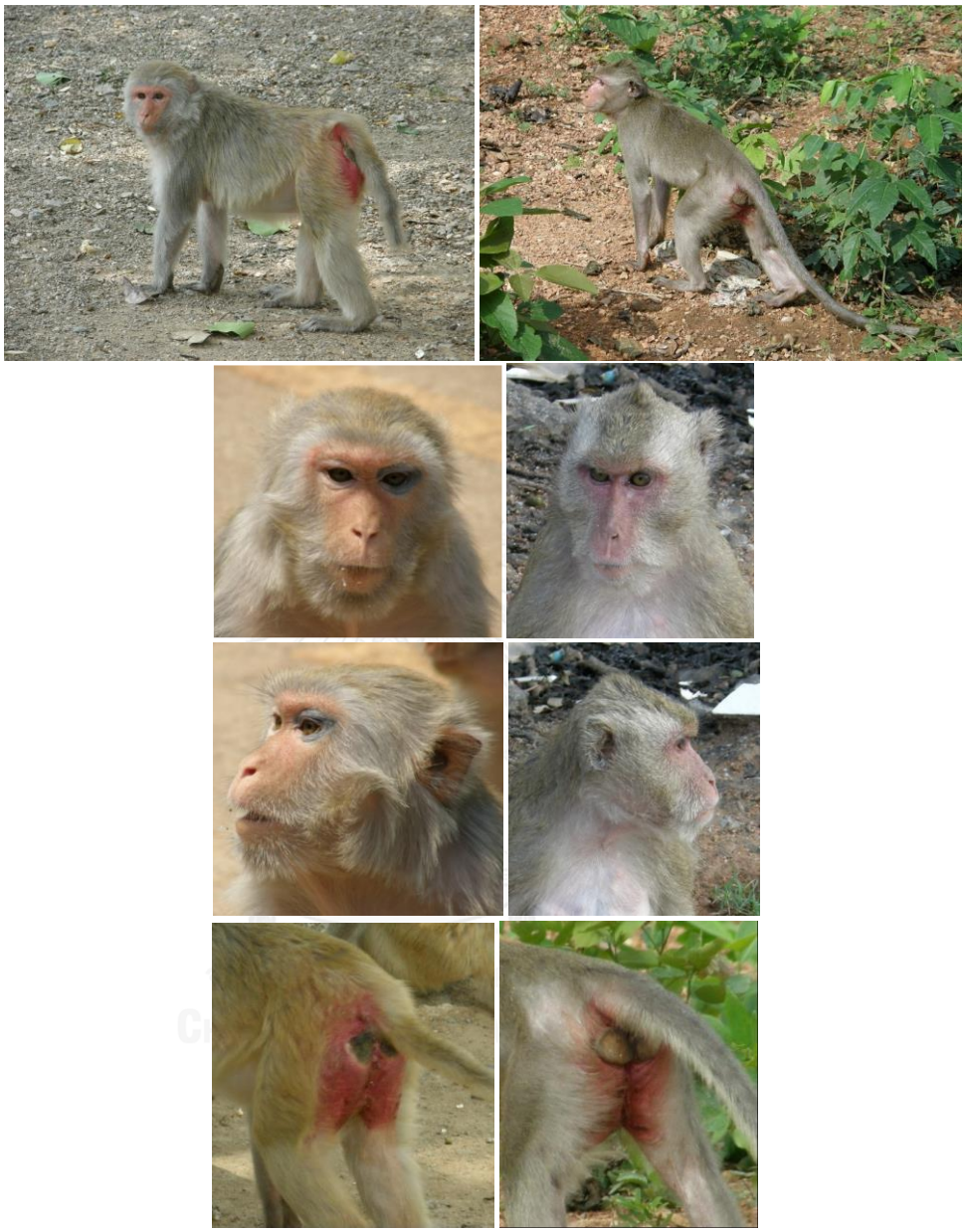

Figure 2. 1 Morphological characteristics of (1) rhesus macaque (left panel) with relatively short tail, with contrast of yellow hue of dorsal palage color between waist and back (bipartite pattern), smooth and posteriorly directed crown hair, infrazygomatic cheek hair, and large area of sex skin reddening and (2) long-tailed macaques (right panel) with relatively long tail, no bipartite pattern, crown hair usually with dark patch and irregular crest, tranzygomatic cheek hair, and relatively smaller area of sex skin reddening (Photos by Janya Jadejaroen).

Their crown hairs usually are smoothly directed posteriorly, and no crest hair (Fig. 2.1). Their hairs on the side of the head (cheek hair) usually form a small crest or whorl below the zygomatic bone near the angle of the jaw (or infrazygomatic crest pattern) (Fooden, 2000; Malaivijitnond and Varavudhi, 2002).

Sex skin reddening in estrous female rhesus macaques usually covers large hindquarters including anogenital areas, legs, thighs, and base of the tail ranging from pale pink to deep red without sexual swelling (Fig. 2.1). The reddening varies according to the phases of ovarian cycle and varies individually (Cleveland et al., 1943; Baulu, 1976; Dixson, 1998; Dubuc et al., 2009). In rhesus macaques, sexual skin color appears to contain general information about the probability of fertility during the ovarian cycle but Dubuc et al. (2009) suggested that only their facial skin color seems to be a reliable visual cue about its timing.

Among rhesus macaques, Chinese ones that distribute in eastern longitude are the heaviest and largest. Indian rhesus macaques distributed in western longitude are lighter and smaller than Chinese ones, while Indochinese ones distributing near and in the hybrid zone between rhesus and long-tailed macaques are the lightest and smallest (Hamada et al., 2005a; 2005b; 2006; 2015). Body weights of male Chinese and Indian rhesus macaques are approximately 20–40% higher than females while that of male and female Indochinese ones are about the same. Body weights of male and female Chinese rhesus macaques are 12.10 ± 1.85 kg and 7.77 ± 1.37 kg, respectively. Those of male and female Indian rhesus macaques are 9.80 ± 2.51 kg and 7.92 ± 1.90 kg, respectively. Body weights of male and female Indochinese rhesus macaques are 5.33 ± 1.25 kg and 4.53 ± 1.27 kg, respectively. Chinese rhesus macaques are 2–5% larger than Indian rhesus macaques (Hamada et al., 2005b). Crown-rump lengths of male and female Chinese rhesus macaques are 585 ± 30 mm and 525 ± 22 mm, respectively. Those of male and female Indian rhesus macaques are 545 ±56 mm and 501± 39 mm, respectively while those of male and female Indochinese ones are 465 ±40 mm and 429± 28 mm, respectively (Hamada et al., 2015).

There is no dimorphism in relative tail length and pelage color in rhesus macaques (Hamada et al., 2015). Chinese rhesus macaques have the shortest relative tail lengths (35.3 ± 4.6%), followed by Indian rhesus macaques (42.5 ± 5.1%), while

Indochinese ones have the longest relative tail lengths (59.9 ± 8.3%) (Hamada et al., 2015). Tail lengths of Chinese, Indian, and Thai (Loei Province, 17°2′N) rhesus macaques, as mentioned above, vary from 36.5%, 44.2%, to 53.9%, respectively (Hamada et al., 2006). Tail lengths of Thai rhesus macaques are up to 70% of their head and body lengths and are 10.0–17.5% greater than those of Indian and Chinese ones (Hamada et al., 2005a; 2005b; 2006).

Contrast of the pelage color in red (a*) and yellow (b*) hues between the lower and upper back is relatively higher and clearer in Chinese and Indian rhesus macaques while it is relatively lower in Indochinese ones (Fooden, 1997; Fooden and Albrecht, 1999; Hamada et al., 2005a; 2005b; 2006; 2015).

Long-tailed macaques

Male long-tailed macaques are approximately 49% heavier and 13% larger (in head and body length) than the females (Fooden, 1995). Adult males weigh 5.36 ± 1.44 kg (range 3.40–12.00, n = 69) while adult females weigh 3.59 ± 0.69 kg (range 2.35–5.44,n = 46). Body weights are comparable in Indochinese and Sundaic populations. Body weight of male and female Indochinese ones are 6.38 ± 1.89 kg (n = 28) and 4.87 ± 0.95 kg (n = 44), respectively. Those of Sundaic males and females are 6.87 ± 1.62 kg (n = 9) and 4.88 ± 1.09 kg (n = 31), respectively (Hamada et al., 2015).

Head and body lengths of adult males and females are 465.6 ± 42.5 mm (range 370–630, n = 238) and 412.0 ± 36.9 mm (range 315–545, n = 161), respectively (Fooden, 1995). Crown-rump lengths of Indochinese and Sundaic long-tailed macaques are comparable. Those of male and female Indochinese macaques are 483 ± 23 mm (n = 28) and 428 ± 18 mm (n = 44), respectively, and male and female Sundaic ones are 479 ± 24 mm (n = 9) and 423 ± 25 mm (n = 31), respectively (Hamada et al., 2015).

Relative tail length of long-tailed macaques is a species-specific character which is usually > 90% (Fig. 2.1) (Hamada et al., 2015). Relative tail length (calculated by HBL) of male and female long-tailed macaques are 117.6± 13.4% (range 69.2–149.5, n = 232) and 116.4± 15.6% (range 70.4–148.4, n = 159), respectively (Fooden, 1995). Indochinese long-tailed macaques have lesser %RTL (calculated by CRL), (< 120%) compared to those Sudaic (> 125%) (Hamada et al., 2008).

Dorsal pelage color is generally similar in both male and female long-tailed macaques but pelage in adult males tends to be longer and sleeker than in other age/sex classes (Fooden, 1995). Dorsal pelage color of this species varies from buffy to yellowish gray to golden brown to reddish brown to blackish. There is no bipartite pattern of the pelage color in both Indochinese and Sundaic long-tailed macaques (Hamada et al., 2008; 2015). In newborn infants, dorsal pelage is blackish (on head, trunk, limbs, and tail), and the facial skin is bare, unpigmented and pinkish (Fooden, 1995).

The crown of long-tailed macaques usually is more brightly colored than the back. The anterior edge of the crown is bordered by a transverse blackish supraorbital streak; hair at the vertex often forms an irregular tuft or crest. In most long-tailed macaques, the lateral facial crest (cheek hair) sweeps upward from near the angle of the jaw to the lateral margin of the crown, passing between the eye and ear (or tranzygomatic crest pattern) (Fig. 2.1) (Fooden, 1995).

Estrous female long-tailed macaques usually show the reddening sex skin from subcaudal down to inguinal region as in rhesus macaques, but usually not cover the full hindquarter area (Fig. 2.1). Sex skin swelling at the base of the tail can be seen in female long-tailed macaques approximately during the ovulation time of the menstrual cycle. The reddening and swelling can vary individually (Engelhardt et al., 2005; Malaivijitnond et al., 2007a). However, sex skin swelling in long-tailed macaques, cannot be used as reliable indicator for the timing of the fertile phase while female sexual behavior can (Engelhardt et al., 2005).

Genetics

Rhesus macaques

There are several studies on genetic characteristics of rhesus macaques. Microsatellite Short Tandem Repeat (STR) markers of rhesus macaques have been developed and used mainly for pedigree testing, paternity analysis, population genetic studies, linkage analysis, and introgression between Chinese and Indian rhesus macaques in captivities (Kanthaswamy and Smith, 1998; Kanthaswamy et al., 2006; 2009; Smith et al., 2000; Hadfield et al., 2001; Kaplan et al., 2005; Rogers et al., 2005;

2006). STR polymorphisms can also be used in managing captive colonies by comparing with other colonies and preventing inbreeding (Hadfield et al., 2001; Andrade et al., 2004; Rogers et al., 2005; Kanthaswamy et al., 2006). In regard to the social structure of male dispersal and female philopatry in rhesus macaques, the Y-chromosome gene was used to indicate male migration and mitochondrial DNA was used for population genetic diversity. It was found in a study on nuclear genome (serum protein and erythrocytic enzyme loci or allozyme polymorphisms) and mitochondrial genomes of rhesus macaques from five regions; Pakistan, India, Burma, southwest China, and southeast China of Melnick and Hoelzer (1992) that rhesus macaque nuclear genetic variation is relatively evenly distributed throughout the populations suggesting no major geographical barriers among them. In contrast, the distribution of maternally inherited mtDNA was diversity which was characterized by local homogeneity and large interpopulation differences. Their results suggested the behaviors of male dispersal and female philopatry in this species.

Mitochondrial DNA fragments of rhesus macaques originated from India and China were used to estimate genetic distances from reconstructed phylogenetic trees by Smith (2005). They found that Indian rhesus macaques were divided into two clusters; one large homogeneous haplogroup with very low levels of nucleotide diversity and no geographic structure, and a smaller haplogroup apparently derived from Burma. The sequences from Burma and eastern and western China were quite divergent from those in the major haplogroup of India. In addition, their results suggested that Indian and Chinese rhesus macaques were reproductively isolated during most, if not all, of the Pleistocene, during which time Indian rhesus macaques experienced a severe genetic bottleneck, and that some gene flow westward into India was subsequently reestablished.

Comparing mtDNA sequences and microsatellite STR loci of Chinese rhesus macaques trapped throughout China to those of captive Indian rhesus macaques by Satkoski Trask et al. (2008) showed that rhesus macaques from western Chinese provinces had more subdivided populations than the eastern Chinese provinces. However, the other study using mtDNA sequences and STR loci detected greater gene flow from west to east of China than *vice versa* (Wu et al., 2013). Mitochondrial DNA

sequences and STR loci were applied for characterizing and distinguishing Indian and Chinese rhesus macaques (Smith, 2005) and assessed admixture between them and indicating the countries of their origins (Smith et al., 2006).

Cross-species amplification of 72 simple sequence repeat (SSR) loci from DNA of six rhesus macaques including from China, India, and Thailand were studied by Morin et al. (1997). They found that, based on the SSR loci, Chinese rhesus macaques were more genetically diverse and unique than were rhesus macaques from India or Thailand.

Mitochondrial DNA loci, STR loci, together with major histocompatibility complex (MHC) alleles of Nepali (Kathmandu) rhesus macaques were compared with those of Indian and Chinese rhesus macaques by Kyes et al. (2006). The result showed a low level of genetic diversity of the Nepali sample. These Nepali rhesus macaques more closely resemble rhesus macaques of Indian origin than those of Chinese origin.

Recently, rhesus macaque genome (Gibbs et al., 2007; Yan et al., 2011) and a lot of single nucleotide polymorphisms (SNPs) have been analyzed in rhesus macaques (Malhi et al., 2007; Fang et al., 2011; Fawcett et al., 2011; Trask et al., 2011; Kanthaswamy et al., 2012; 2014). Malhi et al. (2007) estimating that 60% of those SNPs in rhesus macaques which is of high frequency and could be useful for mapping quantitative trait loci (QTL), genetic management, and studies of individual relatedness, whereas other less frequent SNPs are suggested to be useful as population specific markers for ancestry identification. Ferguson et al. (2007) studied SNPs for distinguishing Indian-origin and Chinese-origin rhesus macaques. They surveyed the 3′ end of 94 genes in 20 rhesus macaques; 10 animals each of Indian and Chinese ancestry and identified a total of 661 SNPs. Of those, 38 SNPs were confirmed as being population-specific. They suggested that the 3′ end of genes is rich in sequence polymorphisms and is suitable for the efficient discovery of gene-linked SNPs. Those ancestral SNPs then could be used for the rapid scanning of rhesus macaques, both to establish animal ancestry and to identify gene alleles that may contribute to the phenotypic differences observed in these populations. Satkoski Trask et al. (2008) also studied SNPs of rhesus macaques and suggested that SNPs loci were more effective at discriminating Indian and Chinese rhesus macaques than STR loci. Kanthaswamy et al.

(2012) used 2,808 SNPs to assess and to ensure pure Indian or Chinese ancestry. Kanthaswamy et al. (2014) tested a set of 384 potential ancestry informative SNPs and identified a final panel of 91 SNPs that can reliably distinguish Indian-origin from Chinese-origin rhesus monkeys. They reported that those genetic tests can be used to determine the ancestral origin of animals and to detect individuals that are hybrids between these two regional populations.

Long-tailed macaques

In long-tailed macaques, human-ABO blood groups have been tested and all AB, A, B and O groups were found, whereas only B group was detected in Chinese and Indian rhesus macaques (Moor-Jankowski and Socha, 1977; 1979; Terao et al., 1979; 1981; Sae-Low and Malaivijitnond, 2003). It was found in a study by Kawamoto et al. (1984) on 29 free-ranging troops of long-tailed macaques of Sumatra, Java, Bali, Lombok, and Sumbawa in Indonesia using 29 kinds of blood proteins that troops inhibiting small islands showed lower variability. Genetic differences between different troops from different islands were higher than those within the same island. This study was in the same line with the mtDNA analysis of long-tailed macaques in west Java, Indonesia (Perwitasari-Farajallah et al., 1999). Thus, protein and mtDNA polymorphisms had been used to characterize a population of long-tailed macaques in Indonesia (Perwitasari-Farajallah et al., 2001).

Later, mtDNA sequences were used to characterize genetic background and to assess country of origins of captive long-tailed macaques from Indochina, Peninsular Malaysia, Indonesia, the Philippines, and Mauritius (Harihara et al., 1986; Smith et al., 2007; Stevison and Kohn, 2008). Tosi and Coke (2007) studied mtDNA and Y chromosomal (SRY and TSPY) genes of Sumatra long-tailed macaques and found that, in regard to mtDNA, they clustered within a clade of insular populations, while Y-chromosomally, some Sumatran animals clustered with their insular stocks while others clustered with continental populations particularly those of Peninsular Malaysia. Osada et al. (2015) sequenced the whole-genome of six long-tailed macaques from Mauritius. Their result showed the extreme population bottle neck that they had overall level of nucleotide diversity of 23% smaller than that of Malaysian long-tailed

macaques together with a reduction of low-frequency polymorphisms. They also confirmed that Mauritius long-tailed macaques were genetically closer to a representative of the Malaysian population than Indonesian ones.

Using mtDNA polymorphism analysis indicated that long-tailed macaques diverged from the common ancestor of rhesus, Formosan (*M. cyclopis*) and Japanese macaques approximately 1.5–3.0 million years before present (BP) and Japanese, rhesus, and Formosan macaques diverged approximately 0.9–1.8 million years BP (Hayasaka et al., 1988). Blancher et al. (2008) divided common long-tailed macaques (*Macaca fascicularis fascicularis*) into 2 clades of haplotype groups; continental (Indochinese and some Indonesian haplotypes) and insular (Philippines, Mauritius and Indonesian haplotypes). Their estimated divergence time between two groups was approximately 1 million years BP and suggested either direct migration from mainland to Indonesia or that remnant lineages form an ancient population genetically close to the mainland were subsequently brought southward to Indonesia < 550,000 year BP. Interestingly, mtDNA analysis of peninsular Malaysia *M. f. fascicularis* indicated that they belonged to Indochinese (but not Sundaic) populations. They estimated that *M. f. fascicularis* have colonized peninsular Malaysia approximately 0.47 million years ago (Abdul-Latiff et al., 2014).

Kikuchi et al. (2007) developed 66 highly polymorphic STR markers applicable for long-tailed macaques using human STR marker information. STR polymorphisms were used for population structure of Tinjil Island, Indonesia (Perwitasari-Farajallah et al., 2010) and peninsular Malaysia (Nikzad et al., 2014). Together with mtDNA, STR loci on autosomes and Y chromosome were used to test samples of Mauritius (Kawamoto et al., 2008). They found that the population had low degree of genetic variability and agreed that the population bred in Mauritius can be used as valuable as model animals for biomedical research because of their genetic homogeneity. However, by testing 96 SNPs of Mauritius long-tailed macaques, Ogawa and Vallender (2014) found substructure among them and suggested a minimum of two to three populations with moderate admixture.

Natural hybridization between rhesus and long-tailed macaques and genetic markers

At present, there have been several studies that showed physiological and genetic evidences of hybridization or introgression between rhesus and long-tailed macaques. Results of a study on human-ABO blood groups among Indochinese rhesus and long-tailed macaques in Thailand showed evidence of hybridization between these two species. Although Indian and Chinese rhesus macaques possessed only B blood group, Thai rhesus macaques had a considerable frequency of major group AB which was similar to that of Thai long-tailed macaques (Malaivijitnond and Hamada, 2008). A study of histocompatibility complex polymorphic (MHC) of Southeast Asian long-tailed macaque (Mafa) populations by Sano et al. (2006) did not only identify 40 novel alleles within exon 2 of Mafa-DPB1 locus by DNA sequencing of 127 individuals but also performed evolutionary and population analyses using these sequences to reveal some population-specific alleles and *trans*-species allelic conservation between long-tailed and rhesus macaques. Creager et al. (2011) studied 127 full-length MHC class II sequences in a group of 12 Indonesian and 12 Vietnamese long-tailed macaques and found 42 novel alleles specific to long-tailed macaques. Apart of that, the 12 alleles of Vietnamese long-tailed macaques were also identical to those previously identified in rhesus macaques.

Using mtDNA and sex-linked Y chromosome markers (testis-specific protein, Y-encoded; TSPY and sex-determining region Y-chromosome; SRY genes) of 44 macaques (18 species), Tosi et al. (2000) showed mitochondrial monophyly, but Y chromosome paraphyly including rhesus and long-tailed macaques with a probable consequence of philopatric females versus dispersing males. Tosi et al. (2002) studied mtDNA and Y chromosome markers (TSPY and SRY genes) of 27 macaques of *fascicularis* species group. Their constructed phylogenies of Y-chromosome also showed a paraphyly among lineages of long-tailed macaques by male rhesus macaques' introgression while the mitochondrial DNA showed monophyly. They suggested an evidence of hybridization or introgression of Y chromosome from rhesus macaques to long-tailed macaque populations in Indochina with Isthmus of Kra as a biogeographic barrier. Using only Y-DNA and mtDNA without autosomal loci, they cited from Fooden (1997) and

Fooden and Albrecht (1999) that morphological studies in particular relative tail length suggested that autosomal genes are diffusing across the species border where the graph of relative tail length against latitude for the two species shows a sharp disjunction where they met in Indochina (ca. 16°N), not a gradual morphocline as expected. This was confirmed later by the study of Hamada et al. (2015) of which their collected specimens were covered the supposed hybrid zone between the two species and the vicinity. Tosi et al. (2003) later analyzed mtDNA, Y chromosome, and autosomal loci of 63 macaques from 19 species and confirmed a contemporary introgression between these rhesus and long-tailed macaques. A study of 13 autosomal, 5 sex-linked microsatellite loci and mtDNA sequence of the parapatric Chinese rhesus and Indochinese long-tailed macaques also suggested no mitochondrial gene flow but unilateral and male-mediated nuclear gene flow from Chinese rhesus macaque to Indochinese long-tailed macaque population (Bonhomme et al., 2009). A study on nuclear DNA sequences (x-chromosomal and autosomal loci) from 19 loci of these two species also supported the hypothesis of unidirectional nuclear gene flow from rhesus into mainland long-tailed macaques which was restricted to mainland Indochina (Stevison and Kohn, 2009).

Using nuclear data from 13 highly polymorphic STR to compare genetic diversity among rhesus and long-tailed macaques suggested significant interspecific nuclear gene flow between the two species on the mainland and marked genetic subdivisions between mainland and island long-tailed macaques due to barriers of their migration (Kanthaswamy et al., 2008). However, Osada et al. (2010) analysed multilocus DNA sequence data of 54 autosomal loci from samples of Indonesian, Malaysian, and Philippine long-tailed and Burmese rhesus macaques. Their results suggested that hybridization between the two species had occurred not only in their natural hybrid zone but also across a wider range for more than 1 million years ago during Pleistocene. After comparing human *CYP3A5* gene sequences from > 100 macaques, they found three nonsynonymous SNPs that were highly differentiated between them. However, for some loci, they found a long-tailed individual had genetically closer to a rhesus macaque than the conspecific siblings. They suggested

that the genetic structure may be very complex because of the past genetic mixture and effect of natural selection on their genomes.

Yan et al. (2011) compared their high-quality draft genome sequences of long-tailed and Chinese and Indian rhesus macaques. They found that all three monkey groups maintained abundant genetic heterogeneity, including millions of single-nucleotide substitutions and many insertions, deletions, and gross chromosomal rearrangements. Their results of genetic divergence patterns also suggested that long-tailed macaque genome has been shaped by introgression after hybridization with Chinese rhesus macaques.

Higashino et al. (2012) studied whole-genome sequences of a male Malaysian long-tailed macaque and identified 9.7 million single nucleotide variants (SNVs) between the Malaysian long-tailed macaques and Indian rhesus genomes. They compared two long-tailed macaques (Malaysian and Vietnamese) and two rhesus macaques (Indian and Chinese) and other previously published genomes and suggested Indochinese long-tailed macaques had been more affected by gene introgression from rhesus macaques. They also identified 60 non synonymous SNVs that completely differentiated long-tailed and rhesus macaque genomes which could be important candidate variants for determining species-specific responses to drugs and pathogens. They also suggested that high-quality whole-genome SNVs sequencing of macaque genome may aid studies including on finding genetic differences that are responsible for phenotypic diversity in macaques.

Various kinds of genetic markers have been studied for introgression and hybridization between long-tailed and rhesus macaques, however, SNPs have recently been used to compare genetic characteristics and discriminate these two species and their hybrids. From a study of Street et al. (2007), 69 and 71 single nucleotide polymorphisms (SNPs) of 20 long-tailed and 20 Indian and Chinese rhesus macaques, 36 SNPs (52%) were found overlapping between the two species. In addition, a majority of 70% SNPs were also presented or conserved in both species. They concluded that their results demonstrated a surprisingly high conservation of SNPs between long-tailed and rhesus macaques, suggesting that the relationship of these two species is closer than that suggested by morphological and mtDNA analysis alone. Satkoski Trask et al.

(2013) studied 2,808 SNP markers in 100 Chinese rhesus macaques, 19 Indochinese (Vietnamese) and 21 Sundaic (Indonesian) long-tailed macaques. Their results showed that the minor allele frequency (MAF) and observed heterozygosity calculated from a sample of Indochinese long-tailed macaques were significantly different from those of Chinese rhesus and Sundaic long-tailed macaques. They concluded that Indochinese long-tailed macaques represented a genetically distinct from Sundaic macaques. Malhi et al. (2011) identified SNPs of 93 individuals of Old World Monkey species cross-species amplification and genotyping. They developed 653 SNPs in rhesus macaques. SNPs identified at the same locus among different species (coincident SNPs) were found in six of the seven species studied with long-tailed macaques exhibiting the highest number (84) of coincident SNPs. They suggested that cross-species amplification and genotyping are useful methods to identify large number of SNPs in closely related species.

Recently, Barr et al. (2011) discovered STAT6 SNP between rhesus macaques (from China, India and Burma) and long-tailed macaques (from Vietnam, Indonesia, the Philippines and Mauritius) using quantitative real-time PCR-based technique. TheStat6 (signal transducer and activator of transcription) gene located on the macaque chromosome 11. They found different SNPs of rhesus and long-tailed macaques at base pair 491 (A for rhesus and G for long-tailed macaques) while hybrid between them (samples from Vietnamese long-tailed macaques) has both A and G alleles. Their result suggested that Stat6 gene can be used for the determination of these two species and the hybrids.

Sexual behaviors and reproductive seasonality

Rhesus and long-tailed macaques both form multimale-multifemale social groups of which females usually live in their natal groups for all their lifetime, so-called female philopatry, and males usually leave their natal groups before sexual maturity or during puberty to join the different groups or spend sometimes alone before joining other groups, so-called male dispersal (Loy, 1971; van Noordwijk and van Schaik, 1985; Manson, 1992; Fooden, 1995; 2000; 2006; Abee et al., 2012).

Behavior is both genetically based and culturally transmitted or learned by an animal to cope with their environment. Behavior of an animal is directed by both internal (e.g., hormones) and external stimuli (e.g., geographical and climatic condition, food supply, behavior of other member/organism, and reproductive history and status). Sexual behaviors, involving with steroid hormones, of an animal reflect not only its evolution but also reproductive status.

Sexual behaviors of female mammals generally include proceptivity, attractivity and receptivity (Beach, 1976). Proceptivity refers to various reactions by the female toward the male, which constitute her assumption of initiative in establishing or maintaining sexual interaction (Beach, 1976). It can be indicated by female attempts at sexual interaction (Wallis, 1982). Attractivity refers to the female's stimulus value in evoking sexual responses by the male (Beach, 1976) which can be indicated by male-initiated attempts (Wallis, 1982). Receptivity is defined in terms of female responses which are necessary and sufficient for the male's success in achieving intravaginal ejaculation (Beach, 1976). This behavior is evident when a female responds positively to male initiation (Wallis, 1982). It also refers as the female's willingness to allow male to copulate and to ejaculate intravaginally (Dixson, 1998). These sexual behaviors link to changes of sex steroid hormones in menstrual cycle. Proceptivity or attractivity is usually high during the follicular phase of the menstrual cycle which assists female in attraction or locating males, whereas her receptivity peaks later in the cycle to coincide with ovulation (Dixson, 1998). Sexual behavior of female long-tailed macaques was reliable indicator for the timing of the fertile phase rather than their sex skin swelling (Engelhardt et al., 2005).

Since rhesus and long-tailed macaques are classified into the same species group, *fascicularis* (Fooden, 1995; 2000; 2006), females of the two species also share similar reproductive characters. Their menstrual cycle lengths are approximately 28–32 days (Nawar and Hafez, 1972; Shimizu, 2008). The follicular phase lasts 8–12 days following with the ovulation (Fooden, 1995). Their gestation lengths are approximately 164–166 days (Jewett and Dukelow, 1972; Silk et al., 1993). Apart from their similarity on reproductive characters, the hybrid offspring between these two species is fertile (Bernstein and Gordon, 1980; Fa, 1989; Satkoski Trask et al., 2013).

Species, in terms of biological species concept of Mayr (1942), are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups. Hybrids between different biological species are commonly less viable and/or fertile than their parents. However, in the case of rhesus and long-tailed macaques, the hybrids between the two species are still fertile. In this case, the species follows the ecological species concept instead. That is "A species is a lineage (or a closely related set of lineages) which occupies an adaptive zone minimally different from that of any other lineage in its range and which evolves separately from all lineages outside its range (Van Valen, 1976)" and/or "A species is a set of organisms exploiting (or adapted to) a single niche" (Ridley 1993).

Distributing in the cooler climate, rhesus macaques are seasonal breeders. Unlike rhesus macaques, long-tailed macaques adapt to living in warmer climate and then relatively weakly develop the reproductive seasonality (or non-seasonal breeders), and females can copulate and give birth all year round with a birth peak during some months (Fooden, 1995; 2000; 2006; Shimizu, 2008). Birth seasons refer to births that occur only in some strict periods or some months of the year, but not in other period, while birth peaks are defined as a tendency to cluster births during some months of the year, or throughout the year (Janson and Verdolin, 2005).

Rhesus macaques copulate and get pregnant during the fall and winter, and give births during the spring and summer (Gordon, 1981). Breeding season of Chinese rhesus macaques in Chongqing (29°N), Yunnan (22°N), and Hainan (18°N) were in October–February (Du et al., 2010), September–January, and November–March, respectively (Fooden, 1995). The breeding period of a population of rhesus macaques living in Loei Province (17°14′N), Thailand was also reported between November–July (Malaivijitnond and Varavudhi, 2002). Long-tailed macaques in Peninsular Malaysia and Sumatra, Indonesia were pregnant all year round with a birth peak during May–July (Kavanagh and Laursen, 1984; van Noordwijk and van Schaik, 1985). Birth period of a population of long-tailed macaques from western Thailand (14–16°N) were from March to May (Fooden, 1971). However, breeding season and birth period can vary locally and annually (van Noordwijk and van Schaik, 1985).

CHAPTER III

USE OF PHOTOGRAMMETRY AS A MEANS TO ASSESS THE HYBRID BETWEEN RHESUS (*Macaca mulatta*) AND LONG-TAILED MACAQUES (*M. fascicularis*)

Introduction

Rhesus (*Macaca mulatta*) and long-tailed macaques (*M. fascicularis*) are the most commonly used non-human primate models for biomedical research (Gibbs et al., 2007). The identification and discrimination of these two species, especially at the hybrid zone in the Indochina Peninsula (15–20°N), can be difficult since their body size, tail length and pelage color are somewhat similar (Fooden, 1995; 2000; Hamada et al., 2006; 2008; Ferguson et al., 2007). In addition to Myanmar, Laos and Vietnam, Thailand is one of the four countries that cover the hybrid zone of these two species. As such, they were grouped as Indochinese rhesus and long-tailed macaques. Although their morphological characteristics in these regions are similar, the principle physiological parameters, such as their complete blood count, serum chemistry (Migot-Nabias et al., 1999), susceptibility to *Plasmodium knowlesi* (Schmidt et al., 1977), ABO blood groups (Moor-Jankowski and Socha, 1979; Malaivijitnond et al., 2008) and reproductive seasonality (Fooden, 1995; 2000) between the two species are different.

Although the natural hybridization between rhesus and long-tailed macaques has been studied, mostly on genetics, for many years (Fooden, 1997; Fooden and Albrecht, 1999; Fooden, 2000; Tosi et al., 2002; Hamada et al., 2006; 2008; Malaivijitnond and Hamada, 2008; Malaivijitnond et al., 2008; Bonhomme et al., 2009), the results are still not clear. A recent hybrid population of these two species, however, has been found in Khao Khieow Open Zoo (KKZ; 13°21′N, 101°06′E) in the eastern part of Thailand (Fig. 1.2) (Malaivijitnond et al., 2011), which is situated within the distribution range of long-tailed macaques but out of that for the rhesus macaques. Individuals of these two species have lived together in the same area of KKZ and some individuals showed mixed-morphological characteristics between rhesus and long-tailed macaques. In this case it appears that the released rhesus macaques joined the

groups of feral long-tailed macaques and have copulated to form this heterospecific population (Malaivijitnond et al., 2011). Thus, researching macaques at KKZ should shed some light on the understanding of hybridization between these two species that has occurred in the past at the hybrid zone (Fooden, 1995; 2000; Hamada et al., 2006; Ferguson et al., 2007).

To identify each species or new species of macaques, the morphological characteristics of tail length and pelage color are often used. The macaque species that inhabits Arunachal Pradesh, India has been recognized as a new species of Arunachal macaques (*M. munzala*) based upon the fact that their relative tail length (RTL; the ratio of the tail length to the head and body length) was intermediate between those of known Tibetan macaques (*M. thibetana*) and western Assamese macaques (*M. assamensis pelops*) (Sinha et al., 2005; Biswas et al., 2011). Rhesus macaques have a bipartite pelage color pattern as a species-specific character, where the lower part of the body shows a more yellowish color compared with the upper part (Hamada et al., 2005a; 2005b). Long-tailed macaques have the %RTL of > 90% (by calculating with head and body length; HBL) (Fooden, 1997). However, to acquire the morphological values of wild or free-ranging macaques, researchers have to capture, anesthetize, immobilize and measure the animals, which can induce stress and sometimes causes injury. It is also time, labor and cost consuming. Besides, it is not always possible to perform this in their natural habitats, including in dense forest and grassland, and so most of the time only photographs could be taken.

Recently, some publications have reported on the determination of the geometric properties of non-human primates from photographs, where this photogrammetric approach has been applied to evaluation of the body length in wild Japanese macaques (*M. fuscata*) (Kurita et al., 2012), sex skin swelling in the wild west African chimpanzee (*Pan troglodytes verus*) (Deschner et al., 2004) and in captive chimpanzees (Mori et al., 2007). However, these photogrammetric studies were used to indicate the nutritional status and reproductive fitness of the individuals and not for species identification. In this study, we used photogrammetry as a tool to assess species identification of two macaque species; the rhesus and long-tailed macaques,

and their hybrids. We hope that the procedures of photogrammetry in this study can be applied to identify other macaque species and their hybrids.

Methods

Validation of accuracy of the RTL values taken by photogrammetry

In total 42 long-tailed macaques from within their distribution range in Thailand (Malaivijitnond and Hamada, 2008; Malaivijitnond et al., 2011) were used in this study. These were composed of one from Ban Pak Nam (BPN; 9° 051′ 46.3″ N, 99° 131′ 56.5″ E), two from Suan Somdej (SSD; 9° 561′ 52.4″ N, 99° 021′ 21.5″ E), seven from Wat Khao Takieb (WKT; 12° 301′ 51″ N, 99° 591′ 09″ E), six from Wat Khao Nor (KN; 15° 571′ 01″ N, 99° 521′ 56″ E), and 14 from KKZ plus 12 monkeys born at the Primate Research Unit, Chulalongkorn University, Thailand of which their parents were also originally from the south, central and northeast of Thailand. Monkeys at the Primate Research Unit were housed in individual cages under standard housing conditions of controlled lighting (12 h light/12 h dark cycle) of the semi-open system. They were fed daily with a monkey chow (Perfect Companion Group Co., Ltd., Samutprakarn, Thailand) in the morning (0900-1000 h), and given fresh fruits in the afternoon (1400–1500 h). Long-tailed macaques were used as subjects for the validation of the tail measurements taken by photogrammetry because their tails are very long and easily detected (Fooden, 1995; Hamada et al., 2006).

To compare the accuracy of the values taken by the photogrammetry with the morphometric measurement, the habituated monkeys living in the recreation parks (BPN and SSD), temple grounds (WKT and WKN) and open zoo (KKZ) were temporarily caught with a net trap (Hamada et al., 2008; Malaivijitnond et al., 2008), anesthetized by intramuscular injection with 10 mg/kg body weight (BW) of ketamine hydrochloride (Sankyo Co., Ltd, Tokyo, Japan), and collected morphological data. Their tail length and crown-rump length (CRL) were measured with an anthropometer (Hamada et al., 2008). After the monkeys were fully recovered from the anesthesia, they were released back to their habitats. The photographs were taken from free-ranging monkeys with a digital camera (Olympus SP-560UZ, Tokyo, Japan), in the distance between 2–10 m during 8 am–4 pm of the day. The photographs were taken on one side of the body

and showed from their crown (vertex of the head), ischial callosity, base of the tail, to the tip of their tails in line. The photogrammetric values of the CRL, taken from the vertex of the head to the ischial callosity, and the tail length (from the base to the tip of the tail) were curve-lineally measured using ImageJ software (Schneider et al., 2012) as shown in Fig. 3.1. The %RTL was calculated as the standardized tail length to CRL ratio x100. Photographs for assessment of pelage color need to be taken when the light condition was not too sunny or too dark or not in the forest shade where sunlight shone inconsistently. An example of photograph taken for measuring pelage color is also shown in Fig. 3.1. The same procedure was also done in the captive monkeys as well.

Validation of the accuracy of the contrast of pelage color values obtained by photogrammetry

Seventeen free-ranging monkeys from KKZ were captured as described in the previous section and used for this study. The morphometric values of the pelage color was directly and quantitatively measured at the waist (suprailiac) and back (interscapular) and expressed by b* for the hue of blue (-60) to yellow (+60) using a digital reflectometer (Color Analyzer Model CR-200, Minolta, Japan) (Hamada et al., 2005b). The photogrammetric values of the pelage color were measured from the taken photographs, three points each at the waist and back of each macaque using the L*a*b* color system in Adobe Photoshop CS Version 8.0 (California, USA). The average of the three values was used in the subsequent calculations for each monkey. The contrast b* values (Cbs*) were calculated from the subtraction of the value at the waist and the back (b*waist - b*back).

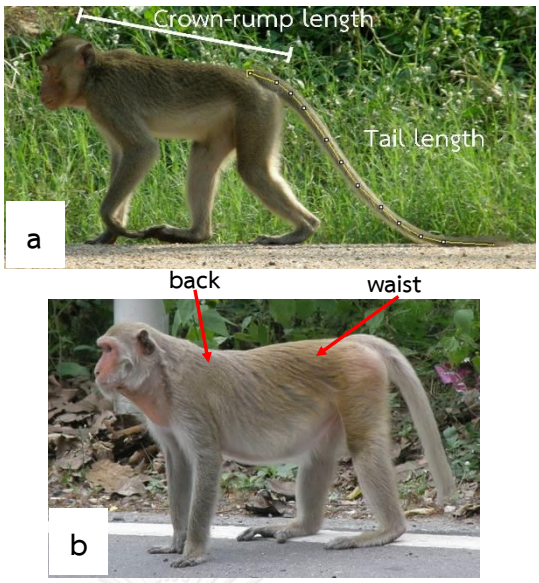

Figure 3. 1 Examples of photographs taken for calculating (a) %RTL from crown-rump length (CRL) and tail length and (b) contrast of yellow hue between waist and back.

Validation of the accuracy of the measurement of the %RTLs and Cbs* from the photographs was tested by determination of the correlation between the morphometric (direct measurement) and photogrammetric %RTLs and Cbs* using Pearson correlation and pair t-test.

Reproducibility and precision of the %RTL values derived by photogrammetry

Fifteen, eight and seven adult monkeys from KKZ, Khao Kaset (KK; 13°07′38.8″ N, 100°55′22.8″ E) and Wat Tham Pa Mak Ho (WTPMH; 17°14′05.6″ N, 101°46′80.8″ E) populations, respectively, were selected. Adult free-ranging long-tailed macaques at KK and rhesus macaques at WTPMH were used as representatives of Thai origin long-tailed and rhesus macaques, respectively. Each monkey was photographed twice in different environment using a digital camera (Olympus SP-560UZ, Tokyo, Japan). The tail length and CRL were then measured and the %RTL was calculated from the photographs as described in the previous section.

Reproducibility and precision of the contrast of pelage color values derived by photogrammetry

Eleven, five and 14 adult monkeys from the KKZ, KK and WTPMH populations, respectively, were photographed twice and then the b* values were measured and the Cb* values were calculated as described in the previous section.

Reproducibility measures of the precision of the %RTLs and Cbs* between the two data sets were tested using Pearson correlation and pair t-test.

Use of photogrammetry as a tool to assess the hybrids between rhesus and long-tailed macaques in the KKZ population

Since only six adult males from 287 individuals of KKZ population were counted in January 2012, and 59 adult females were identified, then the photogrammetry was conducted only in adult female monkeys in this study. Free-ranging adult female long-tailed macaques at KK and rhesus macaques at WTPMH were selected and used as in-group references of Thai origin long-tailed and rhesus macaques, respectively. Monkeys were photographed as already described.

In addition to the %RTL and the Cb* values determined in these three monkey populations (KKZ, KK and WTPMH), the crown hair and cheek hair patterns and the covering area of the sex skin reddening around the ischial callosity, were also recorded and scored as shown in Fig. 2.1 and Table 3.1, respectively. The scoring scale of %RTL, Cb*, crown hair, cheek hair and sex skin reddening of long-tailed macaques, rhesus macaques and hybrids between the two species followed several studies (Fooden, 1995; 1997; Fooden and Albrecht, 1999; Fooden, 2000; Engelhardt et al., 2005; Hamada et al., 2005a; 2005b; 2006; 2008; Malaivijitnond et al., 2007a). Since not all of the taken photographs could be used to determine the above mentioned morphological characters, the number of animals actually used for each measurement of the %RTL, Cb* and other characteristics varied, and are shown in Table 3.2.

Statistical analysis

Results are expressed as the mean ± one standard deviation (SD). The correlation between morphometric and photogrammetric values was determined by Pearson correlation and pair t-test using SPSS version 11.5 for Windows (SPSS Inc., Chicago, USA). Grouping of macaques (18 character states) was done by Multiple Correspondence Analysis (MCA) and by phenetic analysis using Agglomerative Hierarchical Cluster Analysis (AHCA) in XLSTAT (Addinsoft, Inc., New York, USA).

Table 3. 1 The scoring scale used for the %RTL, Cb*, crown hair, cheek hair and sex skin reddening of long-tailed macaques, rhesus macaques and their hybrids. 0 = long-tailed macaque, 4 (for %RTL and Cb*), 2 (for crown hair and cheek hair) and 1 (for sex skin) = rhesus macaque, and the in-between numbers stand for the hybrids between the two species.

Morphological characteristics	Scoring scale				
	0	1	2	3	4
%RTL	> 95.6	87.0–95.6	78.3–86.9	69.6–78.2	< 69.6
Cb*	< 1.33	1.33–1.55	1.56–1.78	1.79–2.01	> 2.01
Crown hair	Dark patch in the middle of the head and sometimes with crest	Mixed	No dark patch. Hairs smooth directed posteriorly		
Cheek hair	Transzygomatic	Mixed	Infrazygomatic		
Sex skin reddening	Red color down to the inguinal part. Usually had a kite shape and swelling at the base of the tail. Reddening rim was usually covered narrower area and within the ischial callosity area.	Red color down to the thigh, and usually had an up-side down U shape. Reddening rim was covered larger area and out of the ischial callosity area.			

Table 3. 2 The numbers of long-tailed, rhesus and KKZ macaques used for each measurement of the %relative tail length (%RTL), relative b* value (Cb*) and other characteristics (the pattern of crown hair, cheek hair and covering area of the sex skin reddening around the ischial callosity).

Morphological characteristics	Number of animals		
	Long-tailed macaque	Rhesus macaque	KKZ macaque
%RTL	30	30	60
Cb*	60	54	60
Other characteristics	60	54	60

Results

Validation and reproducibility of the %RTL values obtained by photogrammetry

The morphometrically and photogrammetrically determined %RTL values of 42 monkeys were very similar with an average of 105.9 ± 18.9 (range 37.8–135.1) and 105.2 ± 18.6 (range 40.6–134.2), respectively. Thus, the mean difference between the two data sets was 2.1 ± 1.94 (range 0.04–7.99) and they were highly significantly correlated (r = 0.989; $p < 0.001$) with no significant difference (t-test, p = 0.1278, n = 42). In terms of the reproducibility of the photogrammetric measurement, the %RTL values of 30 monkeys were close for the first and second measurements, with average values of 100.8 ± 26.9 (range 57.7–139.9) and 100.3 ± 27.6 (range 55.7–143.2), respectively. The mean difference between the two data sets was 4.2 ± 2.9 (range 0.6–11.1), and they were highly significantly correlated (r = 0.983; $p < 0.001$) with no significant difference between them (t-test, p = 0.539). Thus, the %RTL measurement by photogrammetry was reproducible and reliable.

Validation and reproducibility of the contrast of pelage color values derived by photogrammetry

The morphometrically and photogrammetrically derived Cb* values were similar, with the average for 17 monkeys being 0.80 ± 1.93 (range -2.30–3.78) and 0.88 ± 2.00 (range -2.00–4.00), respectively, giving a mean difference between the two data sets of 0.32 ± 0.25 (range 0.06–0.81). The two data sets were significantly correlated (r = 0.98; $p < 0.001$) with no significant difference between them (t-test, p = 0.41). In terms of the reproducibility of the photogrammetric measurement of the Cb* values, that for 30 monkeys were close for the first and the second measurement, with an average of 2.0 ± 4.2 (range -7.7–8.3) and 2.3 ± 1.7 (range -5.7–10), respectively, giving a mean difference between the two data sets of 1.4 ± 1.0 (range 0.3–3.7). The two data sets were significantly correlated (r = 0.914; $p < 0.005$) with no significant difference between them (t-test, p = 0.344).

Use of photogrammetry as a tool to assess the hybrids between long-tailed and rhesus macaques in the KKZ population

The Thai long-tailed macaques (KK population) had an average %RTL and Cb* value of 128.2 ±11.8 (range 95.6–149.6, n = 30) and -0.62 ± 1.51 (range -7–1.33, n = 60), respectively, while the rhesus macaques (WTPMH population) were markedly different with average %RTL and Cb* values of 60.1 ± 5.9 (range 44.8–69.6, n = 30) and 5.62 ± 2.15 (range 2–13.67, n = 54), respectively. For the KKZ population, the 59 assayed macaques had average %RTL and Cb* values of 97 ± 17.01 (range 67.1–135.2) and 1.6 ± 2.39 (range -2.7–6.7), respectively.

Three, three and 59 macaques from the KK, WTPMH and KKZ populations, respectively, were selected for the MCA and AHCA. In the MCA the 59 individuals from the KKZ population were widely distributed and could be divided into five groups based on the morphological variation of L_4R_0, L_3R_1, L_2R_2, L_1R_3 and L_0R_4, respectively, where L stands for long-tailed-like characteristics and R stands for rhesus-like characteristics and the subscript numbers indicated the degree of the species (Figs. 3.2 and 3.3). In addition, the three long-tailed macaques from the KK population fell in

the L_4R_0 group and the three rhesus macaques from the WTPMH population were in the L_0R_4 group. The 19 and 13 individuals of the KKZ population, which were grouped into the L_4R_0 and L_0R_4 groups, respectively, had long-tailed and rhesus morphological characteristics (Fig.3.3a, e), respectively. Five individuals of the L_3R_1 group were morphologically close to those of long-tailed macaques with one or two characteristics of rhesus macaques or intermediate between the two species, while the 12 individuals in the L_1R_3 group were in reversed directions of the L_3R_1 group (Fig.3.3b, d), respectively. The 10 individuals in the L_2R_2 group showed two or three of the five characteristics of one species scored in Table 1 (%RTL, Cb* and crown hair pattern of long-tailed macaques and check hair and sex skin reddening patterns of rhesus macaques (Fig.3.3c). The photogrammetrically derived %RTL and Cb* values for the five groups of the KKZ macaque population are summarized in Table 3.3.

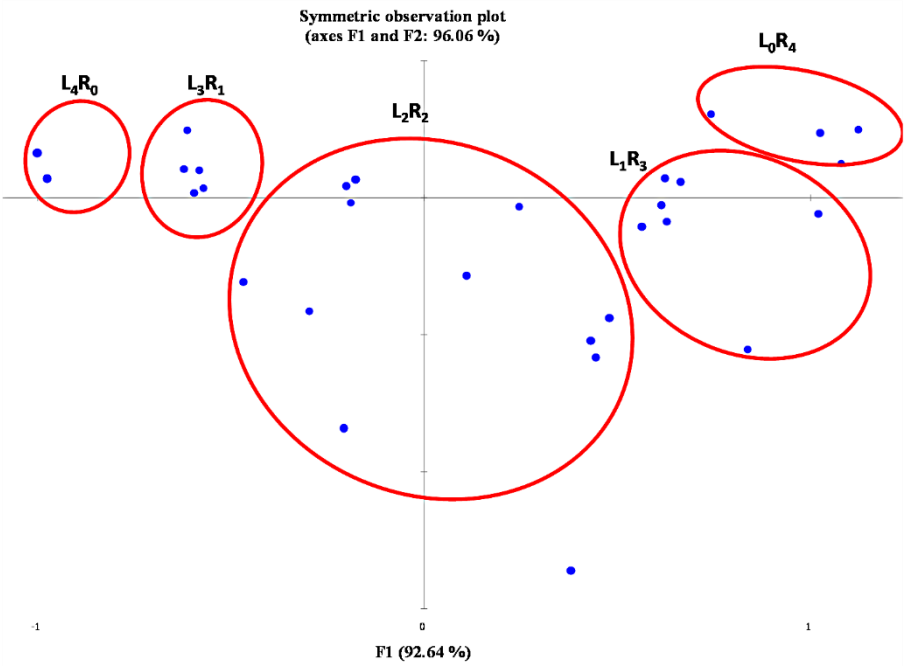

Figure 3. 2 Scatter plots of the morphological characteristics of 59 macaques from KKZ, three long-tailed macaques from KK and three rhesus macaques from WTPMH, based on photogrammetry using MCA. Monkeys were divided into the five groups of L_4R_0, L_3R_1, L_2R_2, L_1R_3 and L_0R_4, where L stands for long-tailed-like characteristics and R stands for rhesus-like characteristics and subscript numbers indicate the degrees of the species.

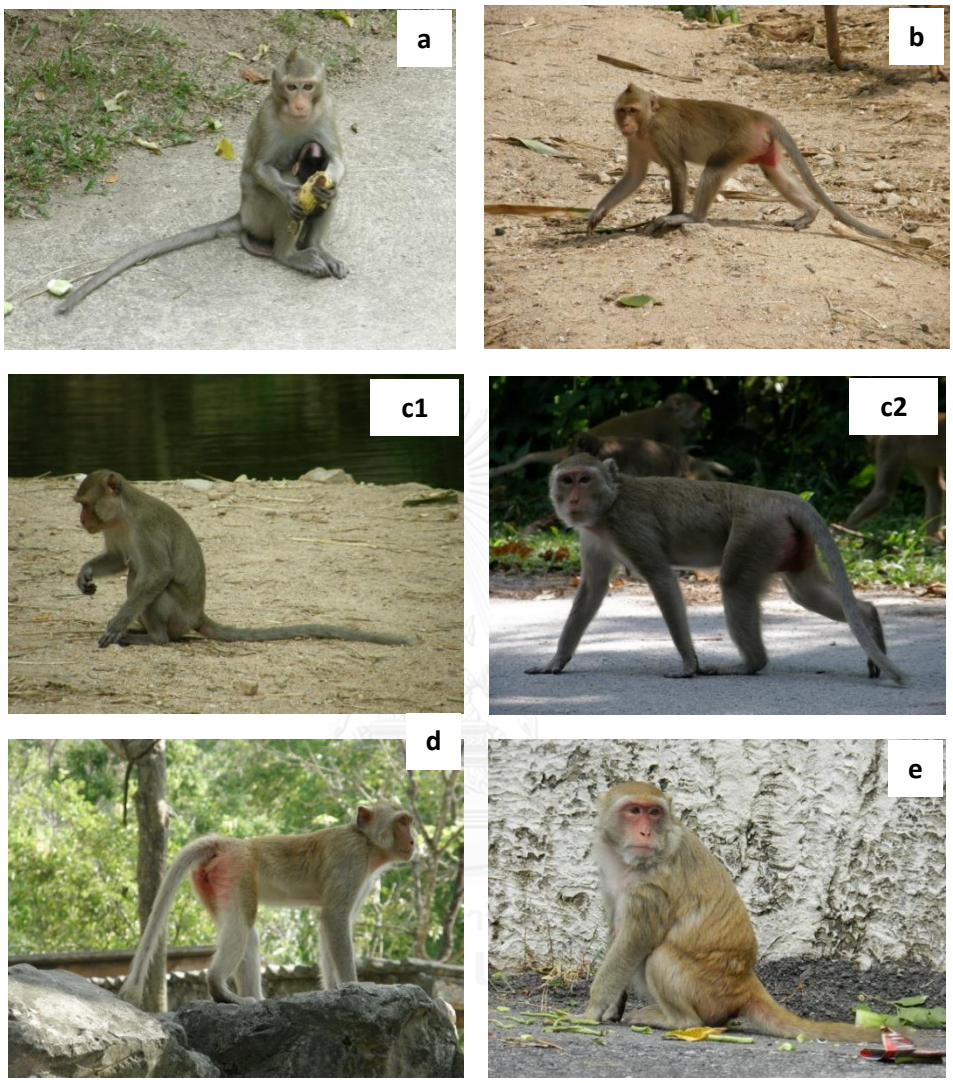

Figure 3. 3 Macaques with morphological characteristics of (a) long-tailed macaques (L_4R_0 group), (b) rhesus-like long-tailed macaques (L_3R_1 group), (c1 and c2) intermediate between long-tailed and rhesus macaques (L_2R_2 group), (d) long-tailed-like rhesus macaques (L_1R_3 group) and (e) rhesus macaques (L_0R_4 group) from the KKZ population as analyzed by MCA.

Table 3. 3 Photogrammetric %RTLs and Cbs* of the five groups of KKZ monkeys as analyzed by MCA. *Three monkeys each from the KK and WTPMH populations were used as in-group references for Thai origin long-tailed and rhesus macaques, respectively. L stands for long-tailed-like characteristics and R stands for rhesus-like characteristics and subscript numbers indicate the degrees of the species.

Group	n	%RTLs			Cbs*		
		Mean ± SD	Min	Max	Mean ± SD	Min	Max
L_4R_0	19(+3)*	112.7 ± 9.2	100.3	135.2	0.4 ± 1.2	-2.3	1.7
L_3R_1,	5	106.9 ± 8.4	98.6	115.8	0.3 ± 1.8	-2.3	2.3
L_2R_2	10	98.2 ± 8.0	86.4	108.2	1.8 ± 1.9	-0.7	5.7
L_1R_3,	12	91.7 ± 4.5	82.9	100.0	3.1 ± 1.5	-0.3	4.7
L_0R_4	13(+3)*	77.4 ± 6.0	67.1	86.2	4.1 ± 1.3	2.3	6.7

The AHCA of 59 individuals from the KKZ population also categorized them into five groups (L_4R_0, L_3R_1, L_2R_2, L_1R_3 and L_0R_4), similar to that observed by MCA but with 18, 11, 7, 10 and 13 macaques, respectively (Fig. 3.4). In the main, the numbers of animals in each group were similar between the MCA and AHCA, except for the rhesus-like long-tailed macaques (L_3R_1) and the long-tailed-like rhesus macaques (L_2R_2). The photogrammetrically derived %RTL and Cb* values of these five groups in the KKZ population are summarized in Table 3.4.

As summarized in Tables 3.3 and 3.4, 27 of the 59 (45.7%) examined individuals in the KKZ macaque population displayed in-between characteristics of long-tailed and rhesus macaques in both the MCA and AHCA and so are likely to be hybrids. This reflected the high intensity of hybridization among the two species in the KKZ area.

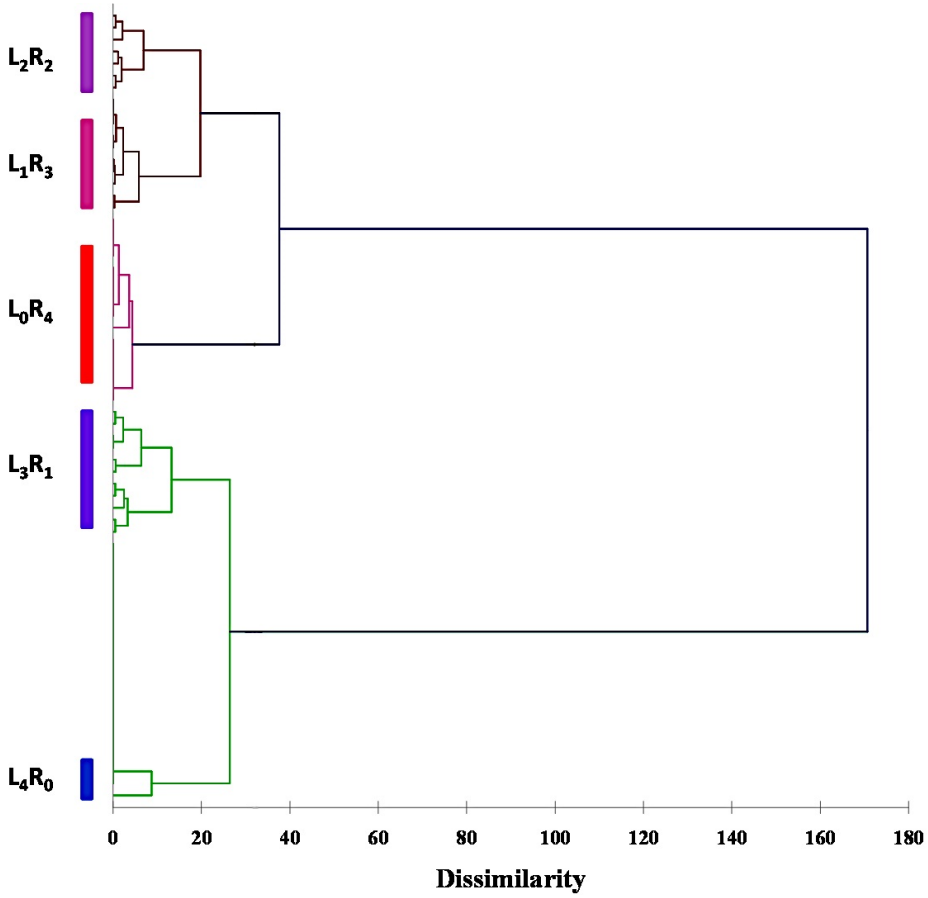

Figure 3. 4 Clustering of 59 macaques from the KKZ population based on photogrammetry using AHCA. Three long-tailed macaques at KK and three rhesus macaques were used as in-group references. Monkeys were divided into the five groups of L_4R_0, L_3R_1, L_2R_2, L_1R_3 and L_0R_4, where L stands for long-tailed-like characteristics and R stands for rhesus-like characteristics and subscript numbers indicate the degrees of the species.

Table 3. 4 The photogrammetric %RTLs and Cbs* of the five groups of KKZ monkeys as analyzed by AHCA. *Three monkeys each from the KK and WTPMH population were used as in-group references for Thai origin long-tailed and rhesus macaques, respectively. L stands for long-tailed-like characteristics and R stands for rhesus-like characteristics and subscript numbers indicate the degrees of the species.

Group	n	%RTLs			Cbs*		
		Mean ± SD	Min	Max	Mean ± SD	Min	Max
L_4R_0	18(+3)*	113.2 ± 9.2	100.3	135.2	-0.6 ± 1.1	-2.3	1.3
L_3R_1	11	101.2 ± 10.0	86.4	115.8	2.1 ± 2.0	-2.3	5.7
L_2R_2	7	99.0 ± 7.4	86.9	108.2	-0.2 ± 0.3	-0.7	0.3
L_1R_3	10	93.0 ± 5.5	82.9	101.0	3.3 ± 0.9	2.0	4.7
L_0R_4	13(+3)*	77.4 ± 6.0	67.1	86.2	4.2 ± 1.3	2.3	6.7

Discussion

Photogrammetry has been widely used in various approaches, such as in medical applications (Pilgrim, 1992), forensic science (Hoogeboom et al., 2009) and land use (Karsli et al., 2009). Photogrammetry is especially popular in medical sciences because of the various benefits that photogrammetry can offer to humanity as a painless and non-invasive means of providing medical practitioners with spatial measurements relating to the human body. It is a tool in disease diagnosis, patient treatment, motion analysis and medical research, and can be used to detect growth, changes before and after treatment or surgery, and defects or abnormalities in organs, such as the face, back, skin, teeth and other parts of the body (Pilgrim, 1992).

Recently, photogrammetry has been applied for body measurements of other animals, such as sponges (Koopmans and Wijffels, 2008), tortoise (Chiari and Claude, 2011), Whale sharks (Rohner et al., 2011), Alaskan moos (Berger, 2012) and apes (Deschner et al., 2004; Machatschke et al., 2006; Breuer et al., 2007; 2012; Mori et al., 2007). Although species identification of old world monkeys, especially macaques, is mostly based on the tail length and pelage coloration (Fooden, 1980; 1995; 1997; Fooden and Albrecht, 1999; Fooden, 2000; Hamada et al., 2005a; 2005b; 2006), the

body size and tail length measurements by photogrammetry has only been applied to Japanese macaques (Kurita et al., 2012). To the best of our knowledge, this is the first study using morphological values taken by photogrammetry to categorize rhesus, long-tailed macaque and their hybrids.

Although measuring the body size of animals from their photographs is easy, of low cost, allows rapid image acquisition, and eliminates the need for direct contact with the subjects, these assets are overshadowed by measurement errors due to the subjective analysis, magnification, parallax, variation in lighting, orientation of the animal's body and subject-camera distances (Farkas et al., 1980). Therefore, before photogrammetry can be used for the routine taxonomic identification of macaque species, its complete evaluation, including its reliability, precision and accuracy is needed (Wong et al., 2008; Metzler et al., 2012). Additionally, to reduce the error caused by the different environments that photographs are taken in, such as variations in the light, distance, skill and examiner-dependent (subjective) interpretations, the use of relative values should ease those problems. Considering the high correlation coefficient of the relative %RTL and Cb* values determined by morphometry with those determined by photogrammetry, and also between the first and the second photogrammetric determinations, this study verified that photogrammetric determination of the %RTL and Cb* values of macaques was as accurate and precise as by direct measurement. The mean repeatability error of the two-time measurements of the photogrammetrically determined %RTL values was only 4%, depending on the posture of the animals. However, that for the Cb* values was markedly higher (65%) because the reflection of the pelage color of monkeys was largely dependent on the light by which the animals were illuminated.

In theory, the absolute values of the macaques' tail lengths acquired from the photographs and not the relative values, should have more meaning, and could be performed by using a laser beam, as previously reported for colobus monkeys (*Procolobus rufomitratus*) (Rothman et al., 2008). However, we have tested it and found that it is difficult for macaques. In non-habituated macaques, the laser spot irradiated on their bodies led to anxiety and stress in the animals, whilst in addition

the process was limited by the slow image acquisition speed, habitat type, and is not always available to field primatologists.

The long-tailed macaques selected for this study are classified, based on geographic distribution, as Indochinese and Malay long-tailed macaques (Fooden, 1995), and are separated by the Isthmus of Kra (10°28′N, 98°48′E). The rhesus macaques belong to the Indochinese or southern group (Fooden, 2000; Hamada et al., 2005a; 2006). The photogrammetrically determined %RTL values of the Thai origin long-tailed macaques in this study were approximately congruent with the morphometrically determined %RTL values of long-tailed macaques reported previously by Fooden (1997) (%RTL > 90) and Hamada et al. (2008) (%RTL < 120% from the north of Isthmus of Kra). Indeed, variation in the %RTL of adult long-tailed macaques followed a latitude cline in accordance with Allen's rule, where the more southerly distributed animals have a longer tail. The average %RTL values were found to be 97.2 ± 10.68 (range 76.1–114.71) for the Indochinese long-tailed macaques and 128.0 ± 15.07 (range 78.8–149.5) for the Malay long-tailed macaques (Fooden, 1995). The %RTL values acquired by photogrammetry in this study also followed this rule, with average values of 122.29 ± 12.3 (n = 27; WKT, KK and KN populations) for macaques at 12°30′–15°57′N, and 127.37 ± 4.03 (n = 3; BPN and SSD populations) for macaques at 9°05′ and 9°5′N.

Likewise, the photogrammetrically determined %RTL values of Thai rhesus macaques from WTPMH (17°14′05.6″ N, 101°46′80.8″ E) determined in this study (60.1% ± 5.9; range 44.8–69.6%) were similar to those directly measured values reported previously (53.9% ± 3.35; range 45–70%) (Hamada et al., 2006) and were also in the range of other rhesus macaques from Thailand and Laos (53.0% ± 4.9) (Fooden, 2000). The photogrammetrically determined %RTL values of the L_4R_0 and L_0R_4 groups categorized by both MCA and AHCA, which represented the long-tailed and rhesus characteristics, respectively, were relatively similar to those morphometrically determined values of Indochinese long-tailed and rhesus macaques, respectively (Fooden, 1995; 1997; Hamada et al., 2005a; 2006).

The photogrammetrically determined Cb* values of the long-tailed macaques from the KK population (0.62 ± (-0.7); range -7–1.33, n = 60) and the rhesus macaques from the WIPMH population (5.62 ± 2.15; range 2–13.67, n = 54) in this study were

roughly congruent with previous reports of the morphometrically determined Cb* values of Thai long-tailed (-0.042 ± 2.49; range -5.4–6.33, n = 96) and Thai rhesus (5.49 ± 2.29; range 1.04–12.26, n = 147) macaques (Hamada et al., 2006; 2008). Generally, the pelage color pattern in rhesus macaques is a bipartite pattern (darkish vertex of the head and back and a vivid reddish yellow at the waist, thigh and legs), which is not found in long-tailed macaques. However, minor differences in this bipartite pattern between Indochinese and Chinese rhesus macaques have been detected (Hamada et al., 2006). Chinese rhesus macaques are darker in the upper half of the body, more reddish in the thigh, and less yellowish in the waist than those of Indochinese rhesus macaques (Hamada et al., 2006). Thus, we used the b* value (blue (-60) to yellow (+60)), instead of the a* value (green (-60) to red (+60)) to distinguish Thai rhesus macaques and the hybrids with long-tailed macaques in this study. Although the Indochinese rhesus macaques had some differences in their morphological (i.e., bipartite pelage color) and genetic characteristics (ABO blood group) from those of the Chinese and Indian rhesus macaques (Hamada et al., 2005a; 2006; Malaivijitnond et al., 2008), all of them were seasonal breeders (Fooden, 2000). In contrast, long-tailed macaques were non-seasonal breeders and can breed throughout the year (Fooden, 1995). As such, observing and scoring the pattern of the sex skin reddening of KZZ monkeys was performed in the breeding season, which is in November–March (Fooden, 2000).

The phenetic analysis based on photogrammetric data increased the understanding of the variation in the morphological characteristics of the hybrids and helped to discriminate them from their parental species (Fig. 3.4). The results of MCA depicted a distribution or gradient of hybrid macaques with various patterns of mixed-morphological characteristics in between the two parental species on a two-dimensional chart (Fig. 3.2). The first axis (with 92.64% of adjusted inertia) clearly gave coordinates (or loadings) of the %RTL, Cb*, crown hair, cheek hair and sex skin reddening area that arranged the monkeys into five groups from L_4R_0 to L_0R_4 in the MCA correspondence map (from approximately -1.0 to 1.0 on the axis). However, in the second axis (3.41% of adjusted inertia), there were some opposite coordinates of morphological characteristics. While the MCA showed a gradient of macaques with

different morphological characteristics, the AHCA grouped those macaques sharing similar morphological patterns from the smallest to largest clusters. The results of both the MCA and AHCA simplified the complicated morphological data and so should be useful for further behavioral, genetic and evolutionary studies.

Because the hybridization of macaques in the KKZ population has only recently occurred, we could still observe some individuals that looked similar to either parental long-tailed or rhesus macaques. Considering the morphological characteristics of the hybrids within L_3R_1, L_2R_2 and L_1R_3 groups, we could not observe the dominated phenotypes of either rhesus or long-tailed macaque over the other species. On the other hand, within the hybrids between rhesus and pig-tailed macaques (*M. leonina*) (Malaivijitnond et al., 2007b), a dark brown and anteriorly narrow crown patch, non-bipartite pattern of pelage color and the thinly-furred tail of the pig-tailed macaques are dominant over the rhesus characteristics. This might be because rhesus and long-tailed macaques belong to the same *fascicularis* species group (Fooden and Albrecht, 1999), but not for the rhesus and pig-tailed macaques. However, we should follow up and analyze the morphological cline of the KKZ hybrid offspring in the subsequent generations (F_1, F_2, F_3,...,F_x). This basic information may help to explain the hybridization process within the natural hybrid zone of these two species (15–20°N) (Fooden, 1995; 2000; Hamada et al., 2006; Ferguson et al., 2007), where it was also postulated that the male rhesus macaques introgressed into the long-tailed population (Tosi et al., 2002). Besides, the intermediate patterns of the monkeys inhabiting the hybrid zone were not usually easily detected and are very complicated because natural hybridization happened slowly and gradually over a very long period of time. Thus, researching the hybrids in the KKZ population should shed some light on hybrid evolution between these co-inhabited two species in the past.

In conclusion, the photogrammetric-based results using relative values on macaques as subjects of measurement were comparable to previous results with direct measurements. As such, this method should be applicable in the identification of macaque species or hybrids where the species identification has mainly relied on the tail-length and pelage color.

CHAPTER IV

A SNP MARKER AT THE STAT6 LOCUS CAN IDENTIFY THE HYBRIDS BETWEEN RHESUS (*Macaca mulatta*) AND LONG-TAILED MACAQUES (*M. fascicularis*) IN THAILAND: A RAPID AND SIMPLE SCREENING METHOD AND ITS APPLICATION

Introduction

Over the past decade, many studies have been conducted on the genetic differentiation and introgression in the Indochina region between rhesus (*Macaca mulatta*) and long-tailed or cynomolgus (*M. fascicularis*) macaques, two of the most commonly used non-human primate models for biomedical research (Tosi et al., 2002; Stevison and Kohn, 2009; Kanthaswamy et al., 2008; 2010; Bonhomme et al., 2009; Osada et al., 2010; Barr et al., 2011; Satkoski Trask et al., 2013). Mitochondrial DNA, Y-chromosome genes testis specific protein, Y-linked (TSPY) and sex determining region Y (SRY), short tandem repeats (STRs) and single-nucleotide polymorphisms (SNPs) (Satkoski Trask et al., 2013) as markers have been widely compared. These two species have high allele sharing and a high level of intraspecific diversity (Bonhomme et al., 2009). Moreover, there are several technical difficulties in these genetic based assessments, such as nuclear insertion for mtDNA (Tosi et al., 2002), allelic dropout in STRs (Malhi et al., 2011), complicated and costly practices in testing STRs and SNPs, or low rates of mutation in TSPY and autosomal genes (Tosi et al., 2002; Osada et al., 2010). Recently, a SNP locus in the interleukin-4 induced signal transducer and activator of transcription 6 (STAT6) was proposed as a candidate diagnostic marker for discrimination between long-tailed and rhesus macaques (Barr et al., 2011) (Barr et al., 2011), where quantitative real-time PCR (qRT-PCR) of this SNP could discriminate 70 rhesus macaques, imported from India, Myanmar and China, from 39 long-tailed macaques, imported from Vietnam, Indonesia, the Philippines and Mauritius. Although macaques from Thailand were not examined in that study, the SNP allele was generally distributed as genotypes homozygous for A and G in the rhesus and long-tailed macaques, respectively, except for three of 19 Vietnamese long-tailed macaques that

showed an A/G heterozygote pattern. Those individuals were postulated to be hybrids, but no additional information was given on their morphology to help support this conclusion (Barr et al., 2011).

In general, SNP analysis has the advantage of being simple in practice, sensitive, less time consuming and suitable for high throughput genotyping, but qRT-PCR is still expensive and is not in widespread use in the countries where these two species occur, especially in the countries where their hybrids occur. Here, a PCR-RFLP method was developed based on the reported STAT6 SNP (Barr et al., 2011). The STAT6 SNP G allele lies within an *Apa*I endonuclease recognition site in long-tailed macaques but not in rhesus macaques (Fig. 4.1). This study examined whether the STAT6 SNP PCR-RFLP products exhibited the predicted single, double or triple bands after *Apa*I digestion for the morphologically defined rhesus, long-tailed and hybrid macaques, respectively, in Thailand (Fig. 4.2).

It has been hypothesized that the hybrids between rhesus and long-tailed macaques occurred over 1.0 million years at the glacial period during the Pliestocene epoch (Tosi et al., 2002; 2003; Osada et al., 2010). The hybrid zone was postulated to be at 15–20° N (Fooden, 1995; Hamada et al., 2006), and the genetic admixture between the two species is considered to extend south to the Isthmus of Kra (Tosi et al., 2002; 2003). In addition, the hybrid zone is likely to result from a unidirectional introgression of male rhesus macaques into the population of long-tailed macaques (Tosi et al., 2002). However, this evolutionary scenario has not been completed, largely due to the paucity of suitable samples from the extant macaques at the hybrid zone.

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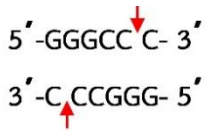

Figure 4. 1 *Apa*I endonuclease recognition site which presented in STAT6 fragments of long-tailed macaques but not in that of rhesus macaques.

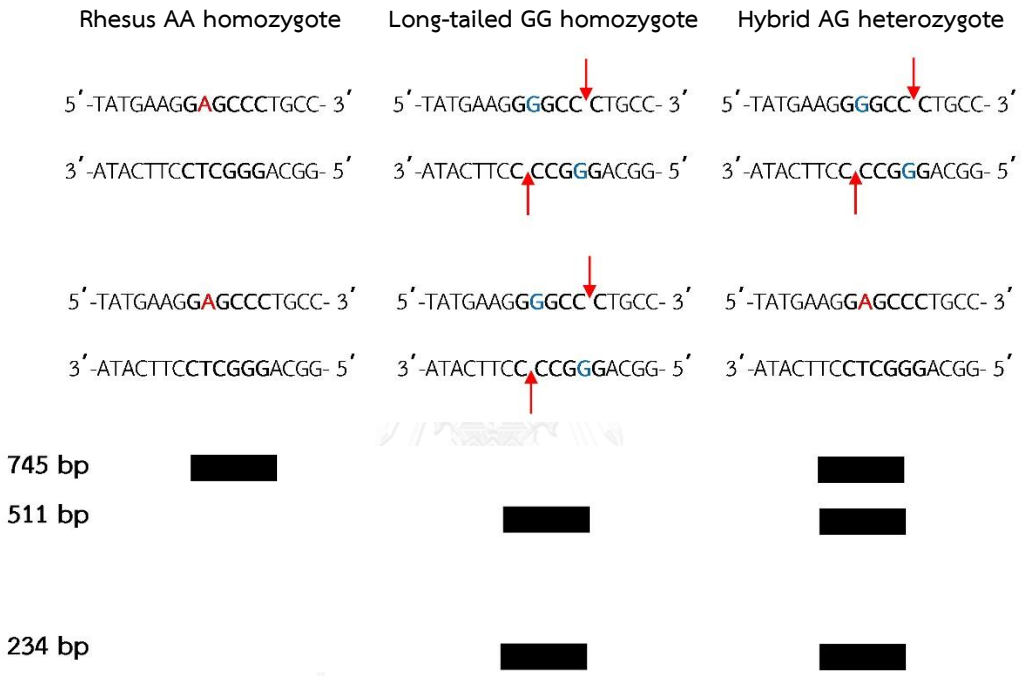

Figure 4. 2 Predicted STAT6 SNP PCR-RFLP products after *Apa*I digestion; single (745 bp), double (511 and 234 bp) or triple bands (745, 511, and 234 bp) for the morphologically defined rhesus, long-tailed and hybrid macaques, respectively.

The Indochina region in Thailand is an interesting area for evolutionary studies, including of rhesus and long-tailed macaques, because it covers the southernmost geographical range of the rhesus macaques and the northernmost range of long-tailed macaques. This encompasses the succession zone of mainland Indochina and Peninsular Malaysia via the constriction of the Isthmus of Kra. However, the current status of hybridization between rhesus and long-tailed macaque remains poorly investigated and resolved, with insufficient evidence to support or refute the

hypothesis and mechanism of their hybridization (Tosi et al., 2002; Kanthaswamy et al., 2010; Barr et al., 2011). Recently, a human-generated hybrid group between released rhesus and free-ranging long-tailed macaques was reported at Khao Khieow Open Zoo (KKZ), Thailand (13°21′ N, 101°06′ E) (Malaivijitnond and Hamada, 2008). This KKZ population is located within the indigenous range of long-tailed macaques, but a small number of both sexes of rhesus macaques (less than 10 individuals) were released here more than 20 years ago (Malaivijitnond et al., 2011). A census performed in January 2012 revealed a total of 500 individuals in three groups (287, 98 and 115 individuals in Groups 1, 2 and 3, respectively) in the KKZ population and the hybrids were restricted only to Group 1. Assuming generation time of 6 years for these macaques, then approximately four generations have been propagated (Osada et al., 2010). However, morphologically, a trace of the parental rhesus macaques is still visible in Group 1, e.g. bipartite pattern of pelage color, infrazygomatic of cheek hair pattern and relative tail length (to body length) < 70% (Jadejaroen et al., 2015). Moreover, changes in the morphological traits in the hybrid group consistent with the gradual increase in the long-tailed macaque-like characteristics over time (Jadejaroen et al., 2015; personal observation). This may reflect the change in the genetic constitution in the Group 1 due to gene flow from long-tailed macaques inhabiting the surrounding areas. As it is well-known that the natural hybridization between rhesus and long-tailed macaques which occurred long time ago could not be traced by the studies of the current hybrid zone. However, the KKZ population depicts the onset of the situation which should have a potential to recall the historical event with the well-known hypothesis of unidirectional gene flow of which rhesus macaques introgressed into long-tailed population. Thus, assessment of the current hybridization process in the KKZ population may give us an insight on modeling the formation and/or maintenance of the hypothesized natural hybrid zone in Thailand. In addition, the previous reported morphological characters of this KKZ hybrid group (Jadejaroen et al., 2015) were compared with the STAT6 genotypes in this study.

Methods

Specimens

Representative samples of wild long-tailed and rhesus macaques in Thailand that cover the Indochinese and Sundaic regions were selected. These were comprised of Indochinese long-tailed macaques from Wangkaew (WK), Sundaic long-tailed macaques from Suansomdej (SSD) (Fig. 4.3) and Indochinese rhesus macaques from Bansang School (BSS). They were temporarily captured using a net trap in 1988, 2006 and 2004, respectively (Kawamoto et al., 1989; Hamada et al., 2008; Malaivijitnond et al., 2008) and anesthetized by intramuscular injection with 10 mg/kg body weight (BW) of ketamine hydrochloride (Sankyo Co., Ltd, Tokyo, Japan). Blood samples were collected and the monkeys were subsequently released back to their habitats when they were fully recovered from the anesthesia. The blood samples were processed by centrifugation at 1,000 xg for 10 min to obtain the buffy coats and then stored at 4°C for the DNA analysis

Fecal samples of the Group 1 macaques from KKZ, which were relatively habituated to humans and consisted of hybrid macaques, were randomly collected in the consecutive years of 2006, 2011 and 2014. Although there might be some possibility of multiple sampling from the same individuals, known adult individuals were mostly followed for the collection of their fecal samples, but unrecognized on their generation, as this group had already been monitored for a sexual behavior study. Additionally, fecal samples of long-tailed macaques from Khao Kaset (KK), which is an appropriate proxy at about 17.6 km from KKZ, were collected in 2011 and used as a reference source to evaluate the genetic constitution of the parental long-tailed macaques that potentially formed and introgressed into the hybrids at KKZ. Rectal cells were swabbed from the surface of fecal samples and kept in lysis buffer (0.5% (w/v) SDS, 100 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0 and 10 mM NaCl) as reported (Hayaishi and Kawamoto, 2006). The numbers of samples used for the analysis and locations of the origin of monkeys are given in Fig. 4.3 and Table 4.1.

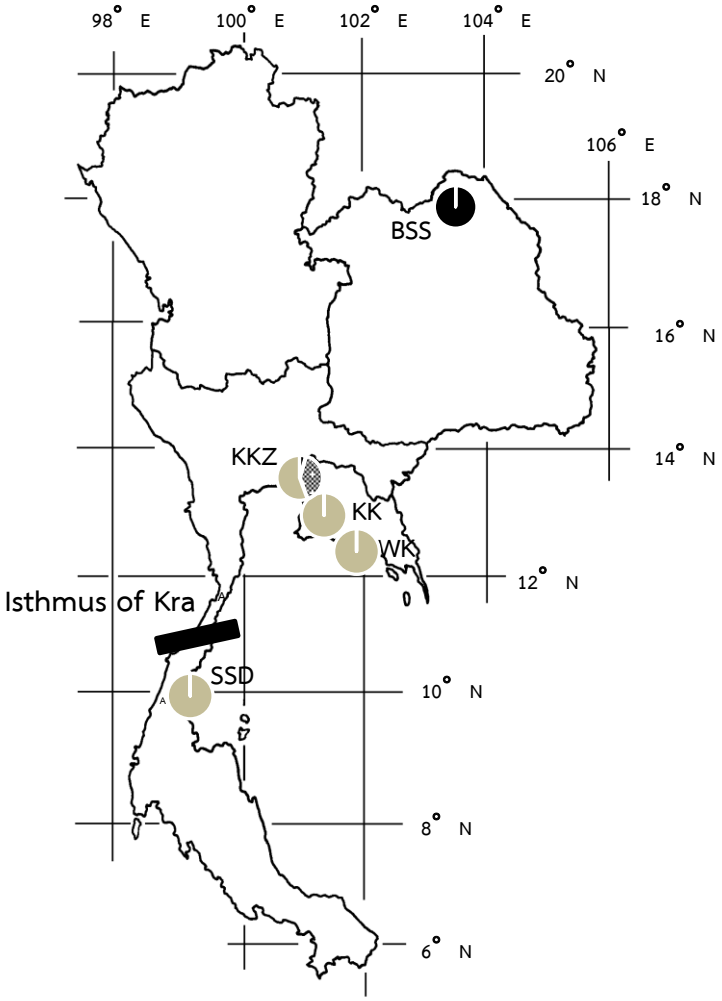

Figure 4. 3 Location of the DNA samples collected and genotype frequency of STAT6 gene of Indochinese long-tailed macaques (WK and KK), Sundaic long-tailed macaques (SSD), Indochinese rhesus macaques (BSS) and the Khao Khieow Open Zoo (KKZ) macaques (where the hybrids between the two species occurred). Grey = GG homozygote, black = AA homozygote, and grey with white dots = AG heterozygote.

Table 4. 1 Location with GPS coordinate, year of collection of the rhesus and long-tailed macaque samples and the allelic frequency of the STAT6 SNP.

Species	Location	GPS (N, E)	Specimen type	Year of collection	No. of samples examined	Observed genotype			Allelic frequency	
						A/A (%)	A/G (%)	G/G (%)	A	G
M. fascicularis	Khao Kaset (KK)	13° 07′, 100° 55′	Feces	2011	8	0 (0%)	0 (0%)	8 (100%)	0	1.0
	Wang Kaew (WK)	12°39′, 101°31′	Blood	1988	9	0 (0%)	0 (0%)	9(100%)	0	1.0
	Suan Somdej (SSD)	9° 56′, 99° 02′	Blood	2004	9	0 (0%)	0 (0%)	9(100%)	0	1.0
M. mulatta	Bansang School (BSS)	17° 51′, 103° 57′	Blood	2006	10	10 (100%)	0 (0%)	0 (0%)	1.0	0
M. fascicularis x *M. mulatta*	Khao Khieow Open Zoo (KKZ)	13°21′, 101°06′	Feces	2006	40	4 (10%)	24 (60%)	12 (30%)	0.40	0.60
	KKZ		Feces	2011	45	2 (4.4%)	18 (40%)	25 (55.6%)	0.24	0.76
	KKZ		Feces	2014	33	0 (0%)	14 (42.4%)	19 (57.6%)	0.21	0.79

The experimental protocol in this study was approved by the Institutional Animal Care and Use Committee of the Faculty of Science in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University, Protocol Review No. 1323010. The field study methods were also in accordance with the guidelines published by the Primate Research Institute, Kyoto University (http://www.internationalprimatologicalsociety.org/docs/Code%20of_Best_Practices%20 Oct%202014.pdf.) and the International Primatological Society (http://www.pri.kyoto- u.ac.jp/research/guide-e2008.html).

DNA extraction from the buffy coats and rectal cells

Genomic DNA samples from the buffy coats were extracted using the conventional phenol-chloroform method (Sambrook et al., 1989). The PCR inhibitors from each rectal cell sample were removed by potato starch (Zhang et al., 2006) and then the DNA was extracted using the Promega (Madison, WI, USA) Wizard SV Gel DNA extraction and PCR Clean-Up System kits (Kawamoto et al., 2013).

STAT6 SNP amplification

The extracted DNA samples were PCR amplified for the STAT6 SNP gene fragment (745 bp) using the KOD FX DNA polymerase (Toyobo Co. Ltd., Japan) and the primers given in Barr et al. (2011) (forward, 5'-AGACACATTTACACTAGAGG-3' and reverse 5'-TCTTTCTTTTCAGATTGTGTA-3'). Each PCR amplification (25 µL total) consisted of 1.0 µL of extracted DNA, 12.5 µL of 2x KOD buffer solution, 5.0 µL of dNTP solution, 0.75 µL of each primer (10 pM), 0.5 µL of KOD FX polymerase and 4.5 µL of pure water. The samples were thermal cycled at 94°C for 2 min followed by 35–45 cycles of 98°C for 10 s, 58°C for 30 s and 68°C for 30 s, and then a 10°C final hold. The amplicons were resolved alongside a 100-bp ladder with 2% (w/v) agarose-TAE gel electrophoresis, and then stained with ethidium bromide, destained in water and visualized under UV transillumination to ensure the specific PCR products were obtained.

Restriction enzyme digestion and genotyping

Each PCR product (2 µL) was digested with *Apa*I in a final reaction volume of 10 µL of reaction buffer at 37°C for 2 h. The digested products (6 µL/well) were resolved, stained and visualized as above. The PCR-RFLP digest of the potentially species diagnostic STAT6 SNP was then subjected to genotyping where homozygotes for the *Apa*I restriction site (long-tailed macaques) yielded two DNA fragments (234 bp and 511 bp) after digestion, homozygotes without this restriction site (rhesus macaques) yielded a single 745 bp band and heterozygotes (hybrids between these two species) will retained three bands (234 bp, 511 bp, and 745 bp) (Fig. 4.2). In order to avoid mistyping caused by incomplete enzymatic digestion, we repeated the genotyping more than two times for all the apparent heterozygotes as well as the two-banded homozygotes.

DNA sequencing of STAT6 gene

To assure that the PCR products obtained were STAT6 fragments and the absence of the other types of SNPs in the restriction site, we randomly selected 10 G/G genotypes (three, three and four samples from KKZ, KK and WK populations, respectively), one A/A genotype and three A/G genotypes from the KKZ macaque population for sequencing using a Genetic Analyzer (ABI PRISM 3100, Applied Biosystems, Foster City, California). After sequencing, the forward and reverse sequences were manually corrected using GENETYX-MAC (version 13.0.11) and SEQUENCE NAVIGATOR (version 1.0.1, Applied Biosystems). The sequences of the rhesus and long-tailed macaques accessed from GenBank (accession numbers JF422670-JF422717) (Barr et al., 2011) were compared with those sequences obtained from the G/G, A/A and A/G genotypes in this study. Sequences were aligned by CRUSTALX (version 2.1, Bioinformatics) (Larkin et al., 2007).

Comparison of the morphological characteristics and STAT6-RFLP genotyped hybrid identification in the KKZ population

The morphological traits of the KKZ macaque population have been followed since 2011 allowing some of them to be individually identified from these traits. Here, 31 fecal samples were collected from individuals with known morphological traits. Of these, seven, seven and 17 individuals had previously been assigned as being long-tailed macaques, rhesus macaques and their hybrids, respectively (Jadejaroen et al., 2015). This was based on morphological characters analyzed by Multiple Correspondence Analysis (MCA) in XLSTAT and Agglomerative Hierarchical Cluster Analysis (AHCA) (New York, USA). Here the results of the STAT6 SNP PCR-RFLP typing were compared with the morphological traits for those 31 monkeys for species identification in order to examine the concordance between genotypes and morphology.

Statistical analysis of the genotype and allele frequencies of the STAT6 SNP in KKZ macaques

Allele frequencies at STAT6 SNP locus were estimated by direct gene counting. Assuming random mating in the study population during the years 2006–2014 following the Hardy-Weinberg Equilibrium, the random mating hypothesis was examined by Fisher's exact probability tests in GENEPOP software (Raymond and Rousset, 1995; Rousset, 2008). Significant changes in the allele frequencies through the study years were examined by Chi-square tests.

Results

Genotyping of Thai long-tailed and Thai rhesus macaques based on the STAT6 SNP PCR-RFLP analysis

All 18 samples of long-tailed macaques collected from the Indochinese region (WK) and Sundaic region (SSD) in Thailand had the G/G genotype, while all 10 samples of Indochinese rhesus macaques (BSS) had the A/A genotype (Table 4.1 and Figs. 4.3 and 4.4). This supports that the STAT6 SNP at position 491 in the amplicon can be used as a diagnostic marker to discriminate long-tailed and rhesus macaques. All eight

long-tailed macaques at KK had the G/G genotype which suggests the absence of A allele in the parental long-tailed population of KKZ and KK.

Evaluation of the genetic admixture between rhesus and long-tailed macaques in the KKZ population

Of the 40 samples of the Group 1 of KKZ macaques collected in 2006, the A/A, A/G and G/G genotypes were found in four, 24 and 12 samples, respectively. Thus, the STAT6 SNP PCR-RFLP genetically confirmed the hybridization between long-tailed and rhesus macaques in this group. The genotype frequency of the STAT6 SNP in the KKZ population during the 8-year period from 2006–2014 was assessed and showed a decreased A/A genotype frequency from 10% to 0% ($p < 0.05$) and an increased G/G genotype frequency from 30% to 57.6%. The decreased allelic frequency of A (from 0.40 to 0.21) and increased allelic frequency of G (from 0.60 to 0.79) were significant ($p < 0.05$) (Table 4.1).

Fisher's exact test for STAT6 polymorphism in the years of 2006, 2011 and 2014 did not reject a random mating hypothesis in the hybrid population ($p = 0.1895$, 1.0000 and 0.2971, respectively).

DNA sequence analysis

The DNA sequences of the 745 bp STAT6 SNP of long-tailed and rhesus macaques from GenBank (accession numbers JF422670–JF422717) (Barr et al., 2011) were aligned with those of the G/G genotype sequences (three, three and four samples from the KKZ, KK and WK populations, respectively), the A/A genotype sequence (one sample from KKZ population) and the A/G genotype sequences (three samples from KKZ population). All the sequences obtained in this study confirmed the successful amplifications of the target STAT6 locus. In addition, no other types of SNPs were observed in the restriction site of *Apa*I and thus it supports that our PCR-RFLP could discriminate A allele from G allele.

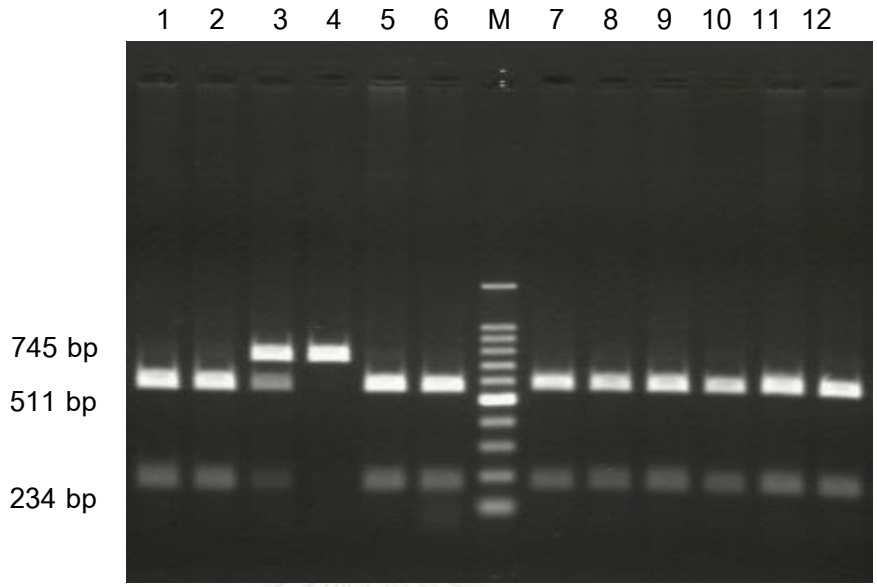

Figure 4. 4 Electropherogram of STAT6 SNP PCR-RFLP digests with endonuclease *Apa*I. Lane nos. 1, 2 and 5–12 indicate long-tailed macaques with two bands of 511 and 234 bp; lane no. 4 indicates rhesus macaques with one band of 745 bp; lane no. 3 indicates a heterozygote or genetic hybrid with three bands of 745, 511 and 234 bp. M = 100 bp DNA ladder. Gel shown is representative of three independent repeats.

Concordance between genotypes and phenotypes of the KKZ macaques

Along with the analysis of the STAT6 SNP locus, seven, 17 and seven KKZ macaques were typed as having a G/G, A/G or A/A genotype, respectively. Compared to the morphological character classification, based on the relative tail length (to the body length), bipartite pattern of the pelage color, and the patterns of the crown hair, cheek hair and sex skin reddening, which classified KKZ macaques into three main groups of long-tailed-like (L_4R_0), hybrid (L_3R_1, L_2R_2 and L_1R_3) and rhesus-like (L_0R_4) macaques (Jadejaroen et al., 2015), the genotypic identification of species using the STAT6 SNP PCR-RFLP pattern was congruent with the phenotypic identification, except for five from 31 individuals that had an A/G genotype but a phenotype of either long-tailed (one) or rhesus (two) macaques, and two monkeys that had the A/A genotype but a hybrid phenotype (Table 4.2). Since the morphological classification is comparable between MCA and AHCA, here only the results of MCA were reported.

Table 4. 2 The concordance between genotypes, based on STAT6 SNP, and phenotypes analyzed by Multiple Correspondence Analysis (MCA) in XLSTAT which was reported previously by Jadejaroen et al. (2015) of the KKZ macaques. *L stands for long-tailed-like characteristics and R stands for rhesus-like characteristics and subscript numbers indicate the degrees of the species. Grey color indicates the discordance between the genotype and phenotype assignments.

Sample no.	Code	Genotype				Phenotype	
		A/A	A/G	G/G	Species	MCA	Species
1	KKZ002			G/G	Long-tailed	L_4R_0*	Long-tailed
2	KKZ003		A/G		*Hybrid*	L_0R_4	Rhesus
3	KKZ005			G/G	Long-tailed	L_4R_0	Long-tailed
4	KKZ006		A/G		*Hybrid*	L_2R_2	*Hybrid*
5	KKZ009		A/G		*Hybrid*	L_1R_3	*Hybrid*
6	KKZ010		A/G		*Hybrid*	L_2R_2	*Hybrid*
7	KKZ015		A/G		*Hybrid*	L_2R_2	*Hybrid*
8	KKZ017			G/G	Long-tailed	L_4R_0	Long-tailed
9	KKZ019			G/G	Long-tailed	L_4R_0	Long-tailed
10	KKZ020		A/G		*Hybrid*	L_2R_2	*Hybrid*
11	KKZ021	AA			Rhesus	L_0R_4	Rhesus
12	KKZ022	A/A			Rhesus	L_0R_4	Rhesus
13	KKZ026		A/G		*Hybrid*	L_2R_2	*Hybrid*
14	KKZ029		A/G		*Hybrid*	L_2R_2	*Hybrid*
15	KKZ030		A/G		*Hybrid*	L_4R_0	Long-tailed
16	KKZ031		A/G		*Hybrid*	L_0R_4	Rhesus
17	KKZ032	A/A			Rhesus	L_2R_2	*Hybrid*
18	KKZ035	A/A			Rhesus	L_0R_4	Rhesus
19	KKZ036	A/A			Rhesus	L_0R_4	Rhesus
20	KKZ037	A/A			Rhesus	L_1R_3	*Hybrid*
21	KKZ038		A/G		*Hybrid*	L_1R_3	*Hybrid*
22	KKZ040		A/G		*Hybrid*	L_1R_3	*Hybrid*
23	KKZ041		A/G		*Hybrid*	L_1R_3	*Hybrid*
24	KKZ042		A/G		*Hybrid*	L_3R_1	*Hybrid*
25	KKZ043			G/G	Long-tailed	L_4R_0	Long-tailed
26	KKZ047			G/G	Long-tailed	L_4R_0	Long-tailed
27	KKZ048	A/A			Rhesus	L_0R_4	Rhesus
28	KKZ049		A/G		*Hybrid*	L_3R_1	*Hybrid*
29	KKZ050			G/G	Long-tailed	L_4R_0	Long-tailed
30	KKZ051		A/G		*Hybrid*	L_1R_3	*Hybrid*
31	KKZ052		A/G		*Hybrid*	L_3R_1	*Hybrid*

Discussion

The STAT6 protein is one of the seven members of the STAT family of cytoplasmic transcription factors. The STAT6 gene is located in humans on chromosome 12 at 12q13.3-q14.1 (Hou et al., 1994), and is comprised of over 19 kb with 23 exons (Godava et al., 2013), whilst it is on chromosome 11 of rhesus and long-tailed macaques and encodes for 847 amino acids (Yan et al., 2011). From our study, it confirms that STAT6 SNP PCR-RFLP can be used to identify and discriminate rhesus macaque (A/A genotype) from long-tailed macaque (G/G genotype). Moreover, in the supposed hybrid group the A/G genotype can confirm the hybrid event. Thus, this simple and inexpensive technique should be applicable for the evaluation of natural hybridization between these two species which occurred in the past and also to test the unidirectional gene flow hypothesis (Tosi et al., 2002; Bonhomme et al., 2009; Stevison and Kohn, 2009; Yan et al., 2011).

Previous studies using the TSPY gene, mtDNA, STRs, SNPs and short putative introgression regions have revealed male-driven gene flow from Chinese rhesus macaques to Indochinese long-tailed macaques (Tosi et al., 2002; Bonhomme et al., 2009; Stevison and Kohn, 2009; Yan et al., 2011), and that the gene flow is unidirectional (Bonhomme et al., 2009). This hypothesis was based upon the (i) male dispersion behavior, (ii) female philopatric behavior (Melnick and Hoelzer, 1992), (iii) male coercion (Stevison and Kohn, 2009) and (iv) reproductive seasonality (Weinbauer et al., 2008). Chinese rhesus macaques are on average 1.3- to 1.4-fold heavier than Indochinese long-tailed macaques of the same gender, with males being heavier than females in each species (7.8 ± 1.4 and 12.1 ± 1.8 kg for female and male Chinese rhesus, and 5.55 ± 1.23 and 8.84 ± 1.36 for female and male long-tailed macaques, respectively (Hamada et al., 2005b; 2008). Thus, it is possible that the heavier rhesus males can coerce the 2.2-fold lighter long-tailed females into copulation, but it is rarely possible in the reverse direction, which leads to asymmetric introgression (Stevison and Kohn, 2009). Consistent with this is that the male-mediated nuclear gene flow from the Chinese rhesus macaque to the Indochinese long-tailed macaques, inferred from the Isolation with Migration model, was approximately 10 migrants per generation (Bonhomme et al., 2009), whilst it was only 0.7 migrants per generation in the reverse

direction of male long-tailed macaques to Chinese rhesus populations (Kanthaswamy et al., 2008). Yan et al. (2011) also noted that the female Chinese rhesus macaques might migrate to male Indochinese long-tailed macaques, but because female rhesus macaque exhibits marked ovarian seasonality and copulate only during ovulation, if female rhesus macaques migrate southward to male long-tailed macaques during non-breeding season, they could not produce the offspring afterwards. Although it is well-known that rhesus macaques are seasonal breeders (Fooden, 2000) and long-tailed macaques can breed throughout the year (Fooden, 1995), it remains unknown if the hybrids between the two species are seasonal or non-seasonal breeders. The evaluation of the sexual behavior of the hybrids at KKZ may help to answer this issue.

Although rhesus and long-tailed macaques are parapartic species (Fooden, 1982), the postulated hybrid zone between them was 15–20°N (Fooden, 1995; 2000; Hamada et al., 2006; Ferguson et al., 2007). Additionally, the hybrids between the two species have been reported to be far beyond the hybrid zone and to reach the geographical barrier at the Isthmus of Kra (Tosi et al., 2002; Sano et al., 2006; Bonhomme et al., 2009; Creager et al., 2011; Ling et al., 2011) or even far beyond the Isthmus of Kra (Osada et al., 2010). A cline in the allelic frequencies of autosomal genes across the geographical distance would support a more ancient and extensive genetic exchange between the two taxa. In an analogous manner to the genetic dilution by distance pattern seen in the rhesus genetic admixture to the long-tailed macaque gene pool across the distribution range of long-tailed macaques (unpublished data), the frequency of introgressed rhesus alleles in the KKZ population followed a dilution over time pattern resulting in low frequencies of rhesus alleles at the present time. As observed in the KKZ population, the allelic frequency of A was decreased (from 0.40 to 0.21) and allelic frequency of G was increased (from 0.60 to 0.79). These temporal changes in the genetic constitution of the KKZ hybrid population probably reflect the shift from a highly heterogeneous state of hybridization to an abundant state of long-tailed genomic genes during the past 8 years. Indeed, similar to the natural hybridization, there were two other groups (Groups 2 and 3) with many long-tailed macaques inhabiting KKZ, whilst the rhesus macaques in regard to their morphology could be seen only in Group 1, and the gene flow between the three groups has

possibly occurred. This was revealed when the targeted adult males in Group 1, which were investigated for their sexual behavior, were found in Groups 2 or 3 (personal observation). Thus, the genotype frequency in Group 1 in the KKZ population did meet assumption of the Hardy-Weinberg Equilibrium and the decreased population frequency of the STAT6 SNP A allele would have resulted from continuous gene flow of long-tailed macaques that carried only the G allele from Groups 2 and 3. Regarding the Fisher's exact test, the disassortative mating within Group 1 might be one of the factors which caused the dilution of the rhesus genetic in the long-tailed population when the time passed by. Based on the decline in the frequency of the STAT6 SNP A allele in the KKZ population, this could imply the unidirectional genetic admixture of rhesus macaques to long-tailed macaques at the natural hybrid zone, as reported previously using 2,808 SNP markers (Satkoski Trask et al., 2013).

The development of DNA markers has become important, for example, for species identification, population genetics and kinship analysis, in non-human primates. Reliable indicators of genetic variation allow for more precise and accurate data processing. Although the STAT6 SNP marker has many advantages of use, such as working well with extremely degraded samples (small size of PCR products), conserved across species and no stutter products, the pattern interpretation is impossible to ascribe in post-F1 hybrids because of backcrossing or chromosome segregation. In this study the PCR-RFLP analysis of the STAT6 SNP had a species identification mismatch (discordance) of 16% (five from 31 individuals) compared to the morphological based identification of species and hybrids, which is because it is not entirely accurate after the F1 hybrids. For example, if the F1 A/G genotype mated to each other to produce offspring (F2), the expected proportion of the offspring having A/A: A/G: G/G genotypes is 1:2:1 but the A/A and G/G genotypes (PCR-RFLP patterns) would be ascribed as pure rhesus and long-tailed macaques, respectively, and not hybrids. On the other hand, if the A/G genotype has been detected, it can identify that they are interspecific hybrids. Though there were some mismatched pairs of genotypes and phenotypes as shown in Table 4.2, only one step mismatch was found. In case of sample Nos. 2 and 16, the one step mismatch were of the samples with hybrid genotype versus rhesus phenotype, No. 15 was of the sample with hybrid genotype but long-tailed phenotype,

and Nos. 17 and 20 were of the samples with rhesus genotype but hybrid phenotype. There was no two-steps or extreme mismatched from rhesus genotype to long-tailed phenotype and vice versa.

However, needless to say, more diagnostic markers are required to obtain a confident result on the degree of hybridization. However, the application of the STAT6 SNP PCR-RFLP pattern analysis could at least provide a preliminary affirmation of the hybrid status for the F1 generation and also the cline of genetic admixture between rhesus and long-tailed macaque. On the other hand, in the captive bred animals used for biomedical research when the non-hybrid monkeys are preferably subjects, the earlier hybrid detection, especially starting from F1 generation, should help to screen the hybrids out of the pure-blood breeding stock. However, we also need to keep in mind that a small sample sizes (≤ 10 samples in each location) of pure long-tailed and rhesus macaques analyzed in this study might cause a biased conclusion. It is plausible that low frequencies of the A and G alleles occur in long-tailed and rhesus macaques, respectively, and may have been missed in this study.

Interestingly, although only single SNP has been used to identify the species and hybrid between rhesus and long-tailed macaques, the congruence between the genetic results with the morphological analysis using Multiple Correspondence Analysis (MCA) is quite high (83.8% or 26 of 31 individuals) (Jadejaroen et al., 2015). Although in the previous report of Jadejaroen et al. (2015), KKZ macaques were classified into five groups of L_4R_0, L_3R_1, L_2R_2 and L_1R_3 and L_0R_4, in this paper we combined the L_3R_1, L_2R_2 and L_1R_3 into a sole group of hybrid macaques. Thus, the STAT6 SNP PCR-RFLP assay should be applicable to identify the rhesus and long-tailed macaque species for the field studies when the researchers could not see wild animals, but can collect only the left behind samples such as fecal or hair samples.

The STAT6 protein regulates immune responses and its activation is required in the interleukin-4 promoted IgE isotype switching (Delphin and Stavnezer, 1995). Some SNPs of STAT6 in humans are association with enhanced serum IgE levels or asthma (Godava et al., 2013). As the G allele was also found in other three species of old world monkeys, *M. nemestrina*, *Papio anubis*, and *Chlorocebus sabaeus* (accession numbers NW_012015577 NC_018162, and NC_023652, respectively), the change from the G

allele in long-tailed macaques to the derived A allele in rhesus macaques may have an effect on some biological functions and thus should be assessed.

In conclusion, a conventional PCR-RFLP protocol was designed and applied for the STAT6 SNP marker for species diagnosis between rhesus and long-tailed macaques in Thailand. This method is simple, robust, efficient, quick and relatively inexpensive. The protocol was useful in the assessment of the spatial distribution or temporal changes in the gene pool of a morphologically hybrid population at KKZ.

CHAPTER V

SEXUAL BEHAVIORS OF HYBRIDS BETWEEN RHESUS (*Macaca mulatta*) AND LONG-TAILED MACAQUES (*M. fascicularis*)

Introduction

Long-tailed and rhesus macaques belong to the *fascicularis* species group that share some biological characteristics including reproductive aspects. They have similar reproductive organs (Fooden, 1995; 2000; 2006). Their ovarian (menstrual) cycle length is approximately 28–32 days (Nawar and Hafez, 1972; Shimizu, 2008; Weinbauer et al., 2008). Their follicular phase lasts approximately 8–12 days following with the ovulation (Fooden, 1995). The gestation lengths of long-tailed macaques are approximately 164.4 days (Jewett and Dukelow, 1972), and about 166.5 days for rhesus macaques (Silk et al., 1993). They both form multimale-multifemale social groups in which females usually spend all their lifetime in their natal groups, so-called female philopatry, and males leave their natal groups or male dispersal during puberty to join the different groups or spend sometimes in male group or alone before joining other groups (Loy, 1971; van Noordwijk and van Schaik, 1985; Manson, 1992; Fooden, 1995; 2000; 2006; Abee et al., 2012).

Although those two species share several biological characteristics, they have different patterns of reproductive seasonality. Long-tailed macaques have relatively weakly developed reproductive seasonality (or so-called non-seasonal breeders) while rhesus macaques have strongly developed one (or seasonal breeders) (Fooden, 1995; 2000; 2006; Shimizu, 2008). Free-ranging long-tailed macaques are known to have copulations, pregnancies, and births throughout the year, however, they also exhibit some peaks of their reproductive events (Fooden, 2000). From 21 broadly distributed natural populations, they gave births every month of the year, but their births tended to peak during some months of the year (Fooden, 1995; 2006). 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 Noordwijk and van Schaik, 1985). Their inferred birth peak was during Mar–May in western Thailand (Fooden, 1971). Birth peaks of long-tailed macaques can vary locally and annually (Fooden, 1995).

Rhesus macaques usually have copulation and pregnancies during the fall and winter, and births during the spring and summer (Gordon, 1981). Of 16 natural populations from Afghanistan, Pakistan, India, Nepal, Vietnam, and China, it is 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). In Thailand (17°14′N), breeding season of rhesus macaques was reported from November to July (Malaivijitnond and Varavudhi, 2002).

Sexual behaviors of female mammals including long-tailed and rhesus macaques composed of three components; proceptivity, attractivity, and receptivity (Beach, 1976) as mentioned in Chapter II. Although there are several reports on long-tailed and rhesus sexual behavior and breeding pattern, no report on that of hybrids between these two species. As mentioned previously that long-tailed and rhesus macaques live in the different climate zones, the hybrids between the two species were observed at 15–20°N. However, to trace the natural hybrid offspring which occurred in the past and study their sexual behavior is difficult. Recently, hybrids between free-ranging long-tailed macaques and released rhesus macaques were recorded at Khao Khieow Open Zoo (KKZ; 13°21′ N 101°06′E), Chonburi Province, Thailand (Malaivijitnond et al., 2011). From an interview of a zoo staff, rhesus macaques had released in the habitat of long-tailed macaques for more than 20 years ago. They have lived together, copulated and produced hybrid offspring afterwards.

The macaque hybrids are usually fertile as their parental species (Bernstein and Gordon, 1980; Fa, 1989; Satkoski Trask et al., 2013), because the chromosomal number of all *Macaca* species is the same, 42 chromosomes (Bernstein and Gordon, 1980). Thus, hybrid offspring in different generations with different degree of morphological

and genetic characteristics of long-tailed and rhesus macaques in KKZ should be representative subjects of this study.

The former two chapters (Chapter III and IV) provided results on morphological and genetic characteristics of the hybrids in KKZ. For morphological characteristic, in regard to relative tail length (%RTL), contrast of dorsal pelage color at lower and upper part of the back (Cb*), crown hair, cheek hair and sex skin reddening pattern, animals were divided into five groups of L_4R_0, L_3R_1, L_2R_2, L_1R_3, and L_0R_4, where L and R stand for long-tailed and rhesus macaques, and the subscript of the numbers indicate the degree of that species (Jadejaroen et al., 2015). For genetic analysis, the SNP at STAT6 locus between long- tailed (G/G), rhesus (A/A), and hybrid macaques (A/G) were used (Jadejaroen et al., in press).

Thus, this study aimed to analyze the sexual behaviors of the known morphological and genetic macaques and to assess whether the hybrids are seasonal or non-seasonal breeders.

Methods

Study site

The study was conducted at KKZ, Chonburi Province, Thailand. The population of long-tailed, rhesus and hybrid macaques were foraging and roaming in approximately 800 ha both in exhibition zones and forested area of the zoo (as shown in Fig. 1.2, Chapter I).

Sexual behaviors

The sexual behaviors recorded were proceptivity (P), attractivity (A), and receptivity (R) and the detailed ethogram of these three behaviors are described in Table 5.1 Proceptivity includes female solicitation, female grooming, affiliative behavior, contact behavior, and female following adult male (Fig. 5.1). Attractivity includes male solicitation, male genital inspection, male grooming, masturbation, male following adult female, and mating (Fig. 5.2). Receptivity is copulation with successful ejaculation (Fig. 5.3).

Subjects

Of three groups (1, 2 and 3) of macaques in KKZ (see Fig. 1.2, Chapter I), Group 1 was chosen for this study because it is composed of many individuals with mixed morphological characteristics of long-tailed and rhesus macaques. Group members was 287 macaques including 60 adult females and 6 adult males. Some females of Group 1 were morphologically rhesus-like and some were intermediate between the two species. Groups 2 and 3 were composed of 98 and 115 macaques, respectively, and no hybrids were counted. Male dispersal among the three groups was usually been observed.

Macaques in the targeted group had been followed, habituated, and identified since the end of 2009. Because of the high rate of male emigration and only 6 male individuals in the group, females were chosen as focal animals. Of 60 adult females, 19 females with analyzed morphological and genetic characters were selected (Table 5.2). Representative photographs of five groups of macaques which were first categorized based on morphological characters and later by genetics (as seen in Table 5.2) are shown in Figure 3.3 (Chapter III). Photographs of all 19 focal females were presented in Figure 5.4. These focal females were randomly chosen mainly based on the reason that they were relatively easy to identify especially when they are in the trees. Each of them was named according to its unique morphological appearances and/or personal characteristics such as colors, scars, habits and others. They were followed and habituated from the end of 2009 to November 2011.

Behavioral sampling

After habituation, sexual behaviors (P, A, and R) of the 19 focal adult females were observed by scan sampling method (Altmann, 1974). Each sampling interval lasted 1 hour. The observations were done between dawn to dusk, for 6–8 days/month, and for 12 consecutive months from December 2011 to November 2012. In each observation interval, all the 19 females or as many as possible were searched. Once one focal female was found, her sexual behavior was observed and recorded. If many focal females appeared in the same place and are not difficult to thoroughly

observe them all, the animal behaviors were scanned from the left to the right hand side and recorded.

Birth observation

Births giving by the 19 females along with the other remaining 41 adult females were recorded monthly from November 2011 to December 2012. Apart from a direct observation of birth given by females seen during the field study, newborn infant (neonate) can be distinguished mainly by its pelage color and facial skin. Pelage color of newborn infants of both long-tailed and rhesus macaques is darker (blackish or dark brown) than adults (Aldrich-Blake, 1980; Fittinghoff and Lindburg, 1980; Higley et al., 1987). Newborn infant's facial skin is bare and pinkish (Rawlins, 1979; Aldrich-Blake, 1980). Dark or blackish neonatal pelage is gradually replaced by paler (brownish or greyish) post-neonatal pelage (Aldrich-Blake, 1980; Fittinghoff and Lindburg, 1980; Higley et al., 1987) and facial skin color begins to change from pinkish to buffy pelage at age about 2–3 months (Higley et al., 1987). Newborn infant depends totally on breast feeding and does not begin to obtain some of their food independently.

Data analysis

Sexual behaviors

P, A and R behaviors of all 19 adult females of known morphological and genetic characteristics and categorized into five groups of L_4R_0, L_3R_1, L_2R_2, L_1R_3, and L_0R_4 were calculated per each observation interval of each month. All P, A and R data were plotted in the same bar chart for each female. The differences of rates of sexual behaviors per observation interval between females in each group were analyzed by Friedman Test (for more than two females) and Wilcoxon Signed Rank Test (for two females) in IBM SPSS Statistics Version 20.0 (IBM, New York).

Pooled data of long-tailed like (L_4R_0), hybrids (L_3R_1, L_2R_2, L_1R_3), and rhesus-like macaques (L_0R_4) were also analyzed for the differences by Friedman Test. Averaged P, A, and R of the three groups were plotted separately. Differences of monthly P, A, and R among the three groups were analyzed by one-way ANOVA.

Birth observation

Births given by the only 19 focal mothers and all 60 (19 focal + 41 remaining) females were plotted and aligned with averaged sexual activities of long-tailed like (L_4R_0), hybrid (L_3R_1, L_2R_2, L_1R_3), and rhesus-like (L_0R_4) groups in each month.

Temperature and rainfall data of the study period were also shown for the comparison with their sexual behavior and birth period.

Table 5. 1 Ethogram of sexual behaviors.

Sexual behavior	Definition
Proceptivity (P)	
Female solicitation	Female invites male by presenting her anogenital region towards the male or moving her head or hand quickly for invitation.
Female grooming	Female cleans the male's fur.
Affiliative behavior	Female stays beside the male without contacting his body.
Contact behavior	Female touches, but does not groom, any part of the male's body including mounting by the female.
Female following adult male	Female keeps following the male within 2-m distance.
Attractivity (A)	
Male solicitation	Male presents his erected penis to the female.
Male genital inspection	Male visually inspects, touches or sniffs of female genitalia.
Male grooming	Male cleans the female's fur.
Masturbation	Male tugs and rubs his penis until it becomes erect with or without ejaculation. The tip will usually be touched with his fingers and will often be sniffed and/or licked.
Male following adult female	Male keeps following the female within 2-m distance.
Mating	Male mounts the female with or without intromission and pelvic thrust and without ejaculation.
Receptivity (R)	
Copulation	Male mounts the female with intromission and pelvic thrust resulted in ejaculation which often involving the female hand clasp, vocalizations by male, female, or both, and ejaculatory plug in vagina of the female.

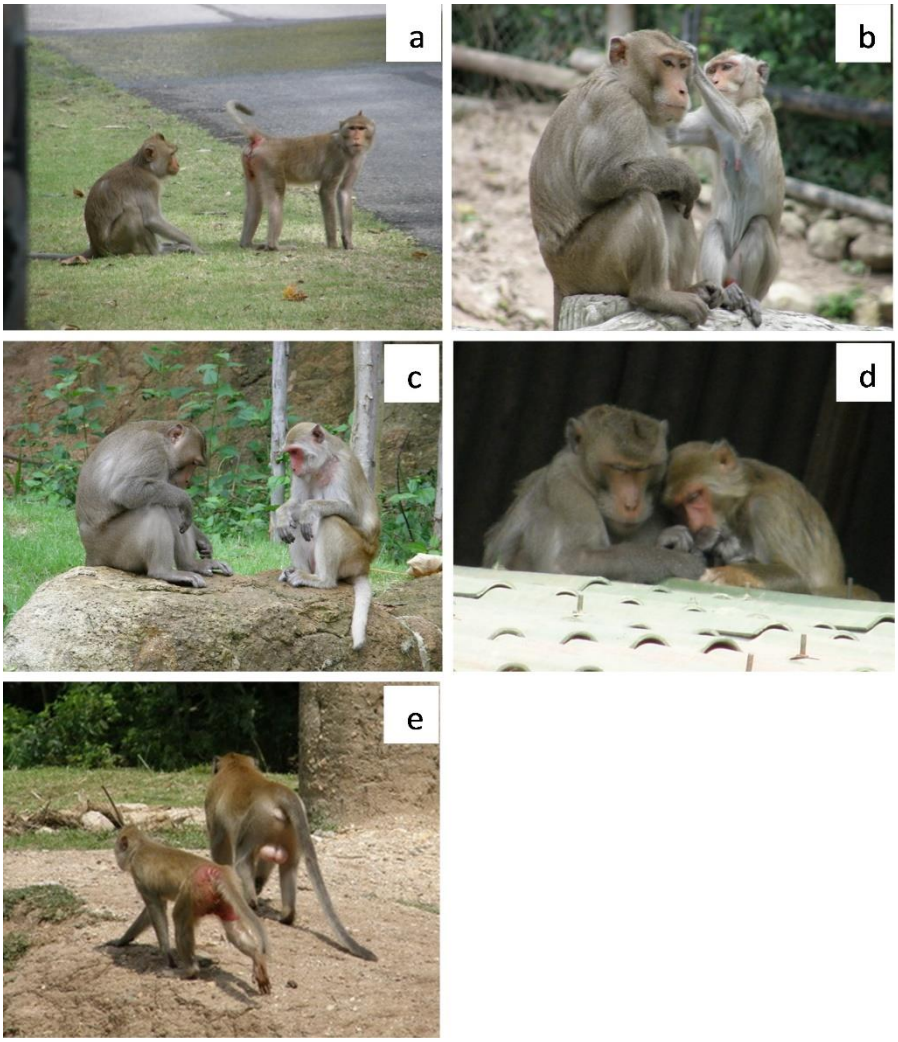

Figure 5. 1 Proceptivity of sexual behavior recorded in this study included (a) female solicitation, (b) female grooming, (c) affiliative behavior, (d) contact behavior, and (e) female following adult male.

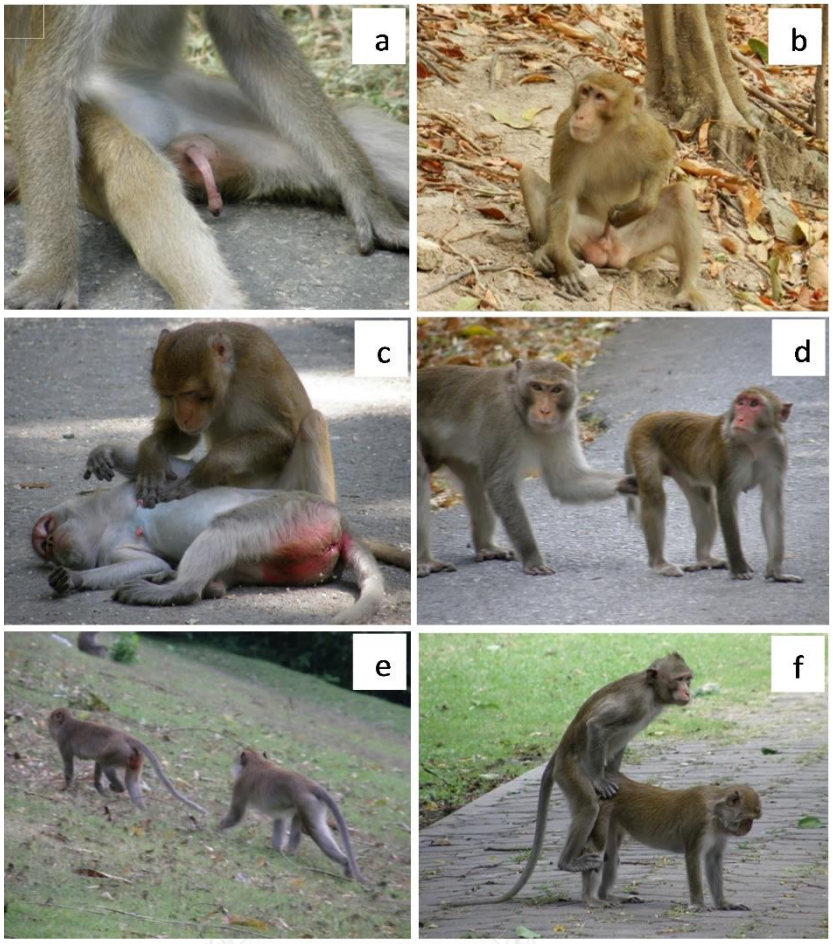

Figure 5. 2 Attractivity of sexual behavior recorded in this study included (a) male solicitation, (b) masturbation, (c) male grooming, (d) male genital inspection, (e) male following adult female**,** and (f) mating.

Figure 5. 3 Receptivity or successful copulation often involves vocalizations by male, female or both, and ejaculatory plug or white semen can usually be observed in vagina of the female.

Table 5. 2 Number of focal animals for sexual behavior study. They were categorized into five groups based on different morphological (Jadejaroen et al., 2015) and genetic characteristics (Jadejaroen et al., in press).

Morphological classification	Genotype composition	Number of focal animals for behavioral study
L_4R_0	G/G	5
L_3R_1	A/G	2
L_2R_2	A/G	4
L_1R_3	A/G	4
L_0R_4	A/A	4
	Total	**19**

L_4R_0

L_3R_1

L_2R_2

L_1R_3

L_0R_4

Figure 5. 4 The 19 focal females for sexual behavioral study categorized into 5 groups based on phenotype and genotype: (i) L_4R_0 morphological characters with G/G genotype, (ii, iii, and iv) L_3R_1, L_2R_2 and L_1R_3 morphological characters with A/G genotype, and (v) L_0R_4 morphological characters with A/A genotype, respectively. L stands for long-tailed like characteristics, R stands for rhesus-like characteristics, and subscript numbers indicate the degrees of the species. (Photos by Janya Jadejaroen).

Results

Sexual behaviors

The total observation of sexual behaviors was 1,014 hours (average 84.5 hours/month, range 72–109 hours/month) from December 2011 to November 2012. Average number of each focal female seen per day was 7.3±1.7 (range 1–14). Average number of each focal female seen per observation hour was 12.4±2.6 (range 1–19). The results showed that 19 female macaques belonging to the L_4R_0, L_3R_1, L_2R_2, L_1R_3, and L_0R_4 group had similar patterns of sexual behaviors throughout the year (Fig. 5.5–5.15, Tables 5.3–5.5). Their sexual behaviors were seen all 12 months of the year with relatively high rates in the early and the end of the year and lowest around March to June 2012. However, R was seen not in all months of the year.

Total sexual behaviors (P, A, and R) of each female (Fig. 5.5–5.14) ranged 0.21–0.95 time/observation interval (mean = 0.66, SD = 0.17). Among three behaviors, P, particularly that males were groomed by females, could be seen in every female, every month of the year, and relatively higher rates comparing to A and R (Fig. 5.5–5.14).

"P" of each female ranged 0.17–0.59 time/observation interval (mean = 0.38, SD = 0.08) or 32.2–100% (mean = 60.2, SD = 13.2) of each female's sexual behavior with average value of each (L_4R_0, L_3R_1, L_2R_2, L_1R_3, and L_0R_4) group (Fig. 5.5–5.14).

"A" were seen in all observations throughout the year (Fig. 5.5–5.14) except one interval of a L_3R_1 female in April 2012 (Fig. 5.7). They ranged 0–0.41 time/observation interval (mean = 0.20, SD = 0.08) or 0–61.9% (mean = 30.4, SD = 9.4).

"R" (copulations) of all the 19 females ranged 0–0.33 time/observation interval (mean = 0.07, SD = 0.08) or 0–36.8% (mean = 9.3, SD = 13.3) of all sexual behaviors (Fig. 5.5–5.14). This behavior was usually not seen or seen in relatively low rates between March and June in most females of all five groups. Interestingly, R of all the four rhesus-like females (L_0R_4) was not seen in April and May 2012 (Fig. 5.13).

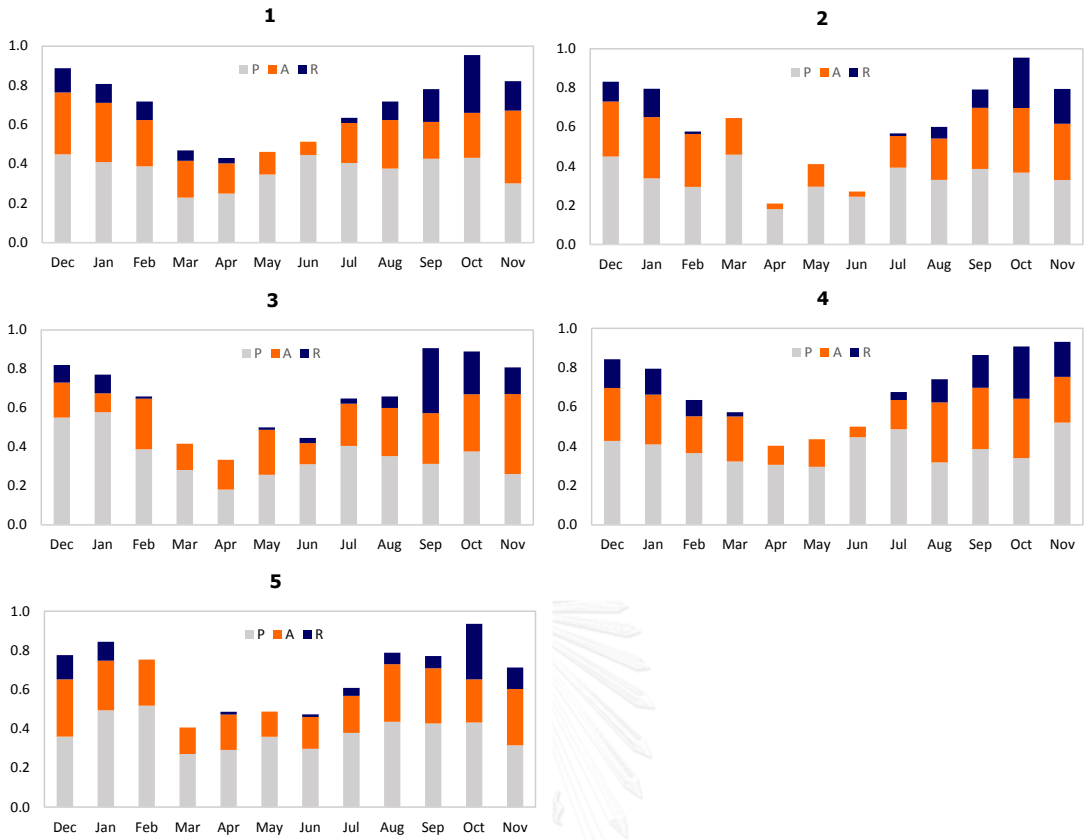

Figure 5. 5 Sexual activities (P, A, and R) per observation interval of five (1–5) L_4R_0 females with G/G genotype.

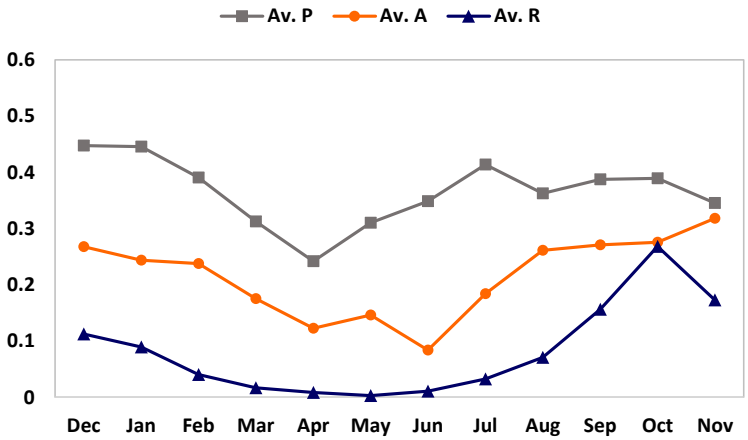

Figure 5. 6 Average sexual activities (P, A, and R) per observation interval of five (1–5) L_4R_0 females with G/G genotype.

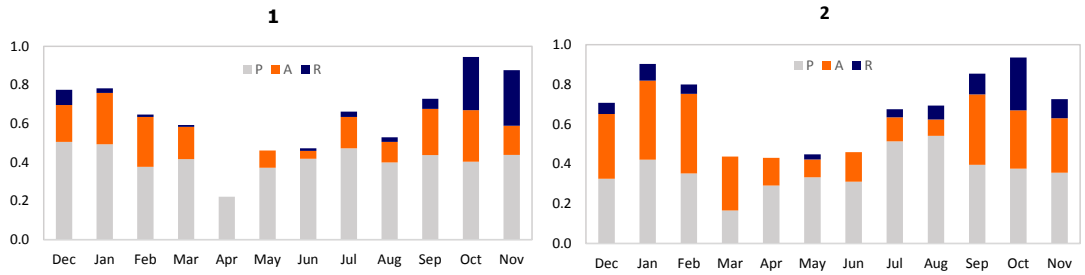

Figure 5. 7 Sexual activities (P, A, and R) per observation interval of two (1–2) L_3R_1 females with A/G genotype.

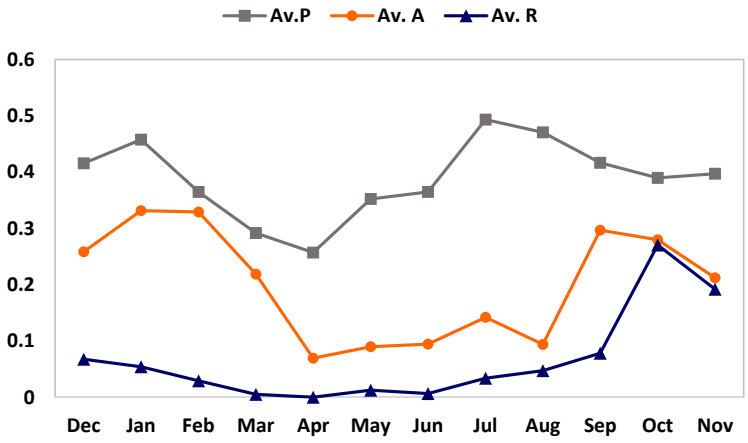

Figure 5. 8 Average sexual activities (P, A, and R) per observation interval of two (1–2) L_3R_1 females with A/G genotype.

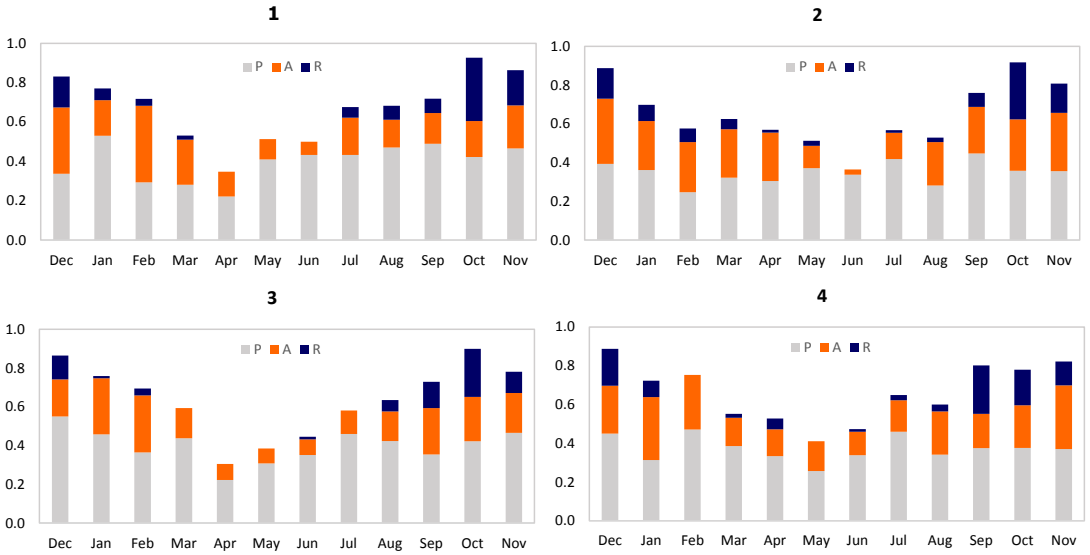

Figure 5. 9 Sexual activities (P, A, and R) per observation interval of four (1–4) L_2R_2 females with A/G genotype.

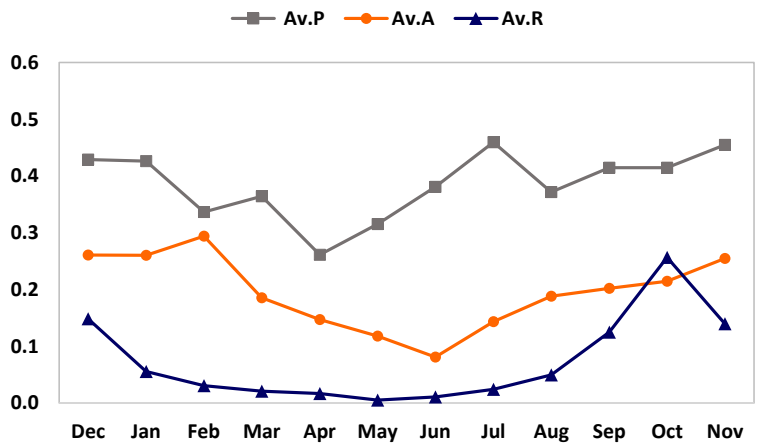

Figure 5. 10 Sexual activities (P, A, and R) per observation interval of four (1–4) L_2R_2 females with A/G genotype.

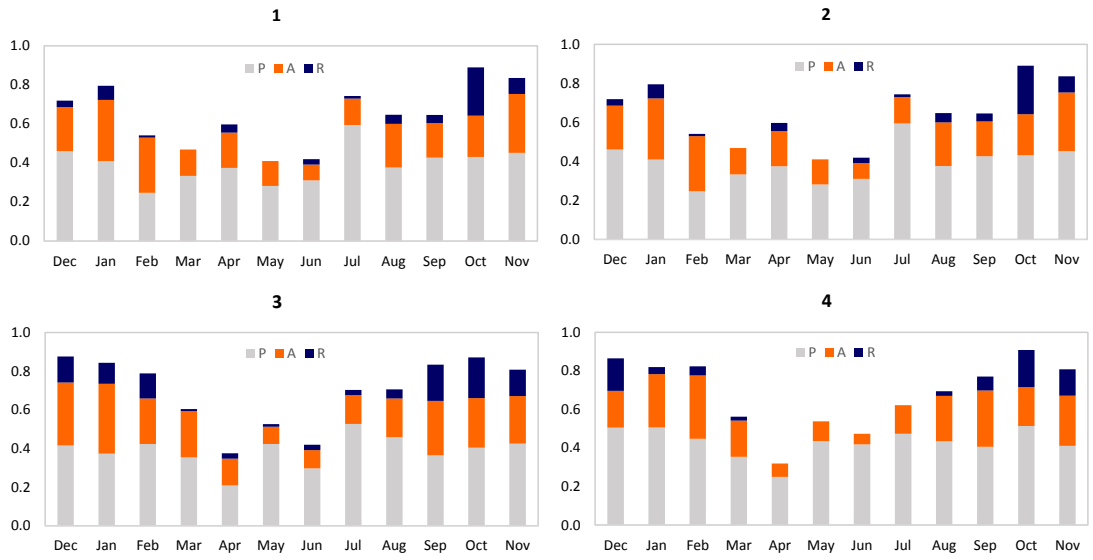

Figure 5. 11 Sexual activities (P, A, and R) per observation interval of four (1–4) L_1R_3 females with A/G genotype.

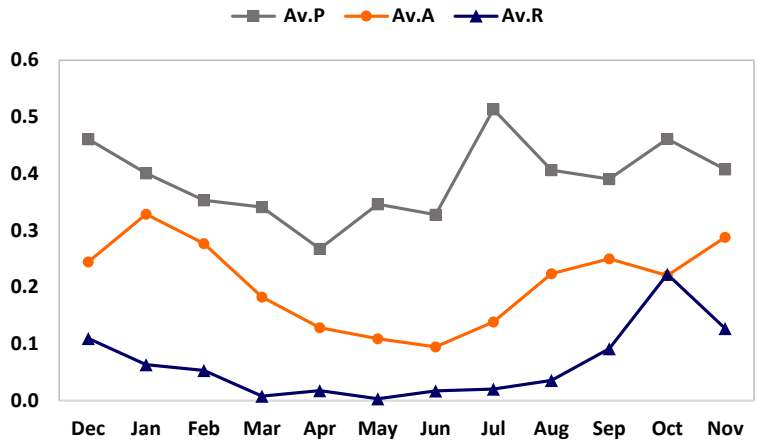

Figure 5. 12 Average sexual activities (P, A, and R) per observation interval of four (1–4) L_1R_3 females with A/G genotype.

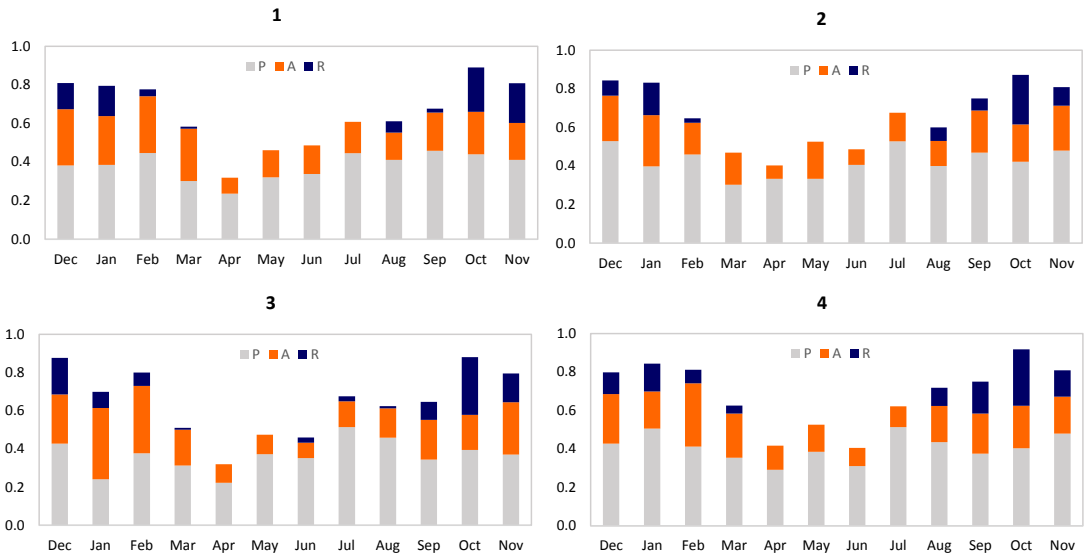

Figure 5. 13 Sexual activities (P, A, and R) per observation interval of four (1–4) L_0R_4 females with A/A genotype.

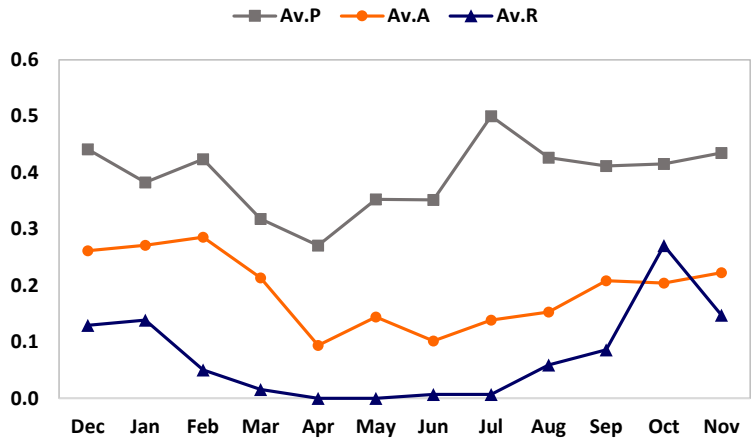

Figure 5. 14 Average sexual activities (P, A, and R) per observation interval of four (1–4) L_0R_4 females with A/A genotype.

There was no statistically significant difference of sexual behaviors among females in each group except P of L_1R_3 (p = 0.003) and A of L_3R_1 (p = 0.013) (Table 5.3). Then data of females with long-tailed (L_4R_0), hybrid (L_3R_1, L_2R_2, L_1R_3), and rhesus (L_0R_4) characters were pooled and the statistically significant differences were shown in Table 5.4. P, A, and R of L_4R_0 and L_0R_4 were not significantly different as previously mentioned. While P values of each of the 10 females in L_3R_1, L_2R_2, and L_1R_3 were significantly different (p = 0.012), while A and R of these females were not. By excluding L_1R_3, six females of L_3R_1 and L_2R_2 showed no significant difference of their P (p = 0.093).

In regard to the statistical analysis of frequencies of P, A and R behaviors in Table 5.4, the data were plotted and shown in Fig. 5.15. For P, four groups of long tailed-like (L_4R_0), hybrid (L_3R_1 and L_2R_2), hybrid (L_1R_3), and rhesus-like (L_0R_4) macaques were separately drawn. Averaged P, A, and R of all the three groups ranged 0.24–0.51 (mean = 0.38, SD = 0.06), 0.08–0.32 (mean = 0.20, SD = 0.07), and 0–0.27 (mean = 0.08, SD = 0.08), respectively (Fig. 5.15). Overall, the profiles of values of P, A, and R of long tailed-like, hybrid, and rhesus-like females were similar all year round. There was no significant difference among groups in P (Table 5.5). For A, significant differences were detected in July (p = 0.017), August (p = 0.008), and October (p = 0.033) where long tailed-like females (L_4R_0, G/G) had the higher rate of A during that time points, following by hybrid and rhesus-like females, respectively. For R, there was no significant difference among the three groups, except in January (p = 0.001) where R of rhesus-like females (L_0R_4, A/A) was at the highest rate, following by the long tailed-like and hybrid females (Table 5.5, Fig. 5.9).

Table 5. 3 *p* values (Friedman and Wilcoxon Signed Rank Tests) of differences of P, A, and R among females of each group (* significance level $p \leq 0.05$).

Group (no. of female)	P	A	R
L_4R_0 (5)	0.399	0.990	0.111
L_3R_1 (2)	0.146	**0.013***	0.396
L_2R_2 (4)	0.103	0.235	0.241
L_1R_3 (4)	**0.003***	0.774	0.083
L_0R_4 (4)	0.061	0.676	0.151

Table 5. 4 *p* values (Friedman Test) of differences of P, A, and R among females of long tailed-like (L_4R_0), hybrid (L_3R_1, L_2R_2, L_1R_3 or L_3R_1, L_2R_2), and rhesus-like (L_0R_4) characteristics (* significance level $p \leq 0.05$).

Group (no. of females)	P	A	R
L_4R_0 (5)	0.399	0.990	0.111
L_3R_1, L_2R_2, L_1R_3 (10)	**(0.012*)**	0.068	0.059
L_3R_1, L_2R_2 (6)	0.093	-	-
L_0R_4 (4)	0.061	0.676	0.151

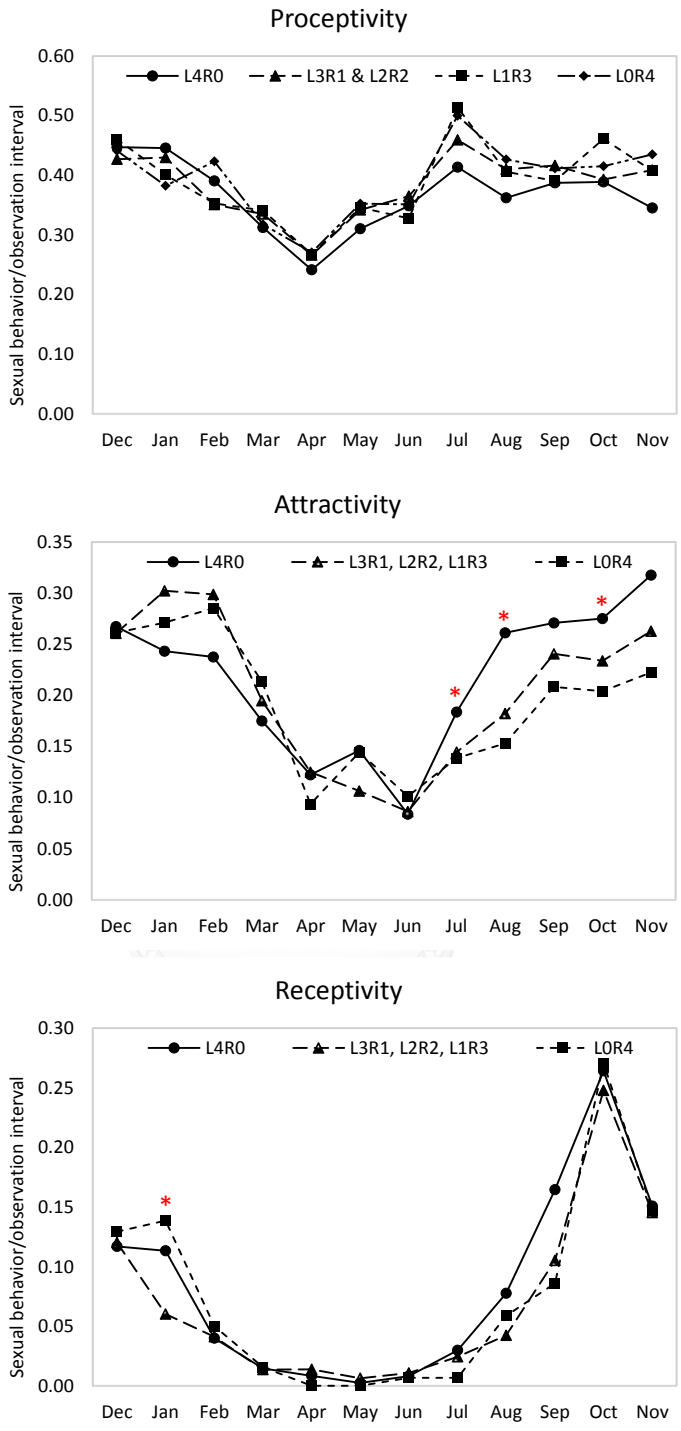

Figure 5. 15 Rates of P, A, and R behaviors of female macaques with long tailed-like (L_4R_0; G/G), hybrids (L_3R_1, L_2R_2, L_1R_3; A/G), and rhesus-like (L_0R_4; A/A) characteristics from December 2011 to November 2012 (* significant differences among the three groups.).

Table 5. 5 *p* values (One-way ANOVA) of differences of the rates of P, A and R behaviors of female macaques with long tailed-like (L_4R_0; G/G), hybrids (L_3R_1, L_2R_2, L_1R_3; A/G), and rhesus-like (L_0R_4; A/A) characteristics from December 2011 to November 2012 (* significance level $p \leq 0.05$).

Month	P	A	R
Dec	0.966	0.991	0.930
Jan	0.701	0.308	**0.001***
Feb	0.431	0.210	0.889
Mar	0.730	0.472	0.981
Apr	0.842	0.674	0.413
May	0.726	0.063	0.415
Jun	0.988	0.835	0.928
Jul	0.073	**0.017***	0.129
Aug	0.175	**0.008***	0.060
Sep	0.879	0.231	0.284
Oct	0.440	**0.033***	0.571
Nov	0.270	0.070	0.974

Birth observation

During the 12-month observation, 19 focal females gave births to 20 infants (one female of L_2R_2 gave twin) in three months; March, April and May 2012 with 6 (30%), 12 (60%), and 2 (10%) infants, respectively (Table 5.6). One infant born in March 2012 was disappeared a few days after birth.

By including births of the other 41 females in the same group, all of the 60 females gave births to 59 infants over a 5-month period from February to June 2012 with 1 (1.7%), 17 (28.8%), 26 (44.1%), 13 (22.0%) and 2 (3.4%) infants in each month, respectively (Fig. 5.16), but peaked during March to May. After giving births, all the sexual activities (P, A, and R) of females were relatively low, and increased again in June 2012 (Fig. 5.16–5.17). It is shown in Figure 5.17 that rates of copulation (R) were highest from September to November with the birth peak during March to May.

Figure 5.17 shows the data of temperature and rainfall at Chonburi Meteorological Station, from December 2011 to November 2012. Monthly rainfall ranged 0–347.7 mm (mean = 113.2 mm). The total rainfall was 1,358 mm. Monthly mean temperatures ranged 26.7°C in December 2011 to 30.9°C in April 2012 (mean = 29.1°C). It could be classified into cool dry (December–February), early-rainy (March–May), mid-rainy (June–August) and late-rainy (September–November) seasons. During this study, KKZ macaques' receptivity reached to the highest rate in October during late rainy season (September–November) and their births were highest during the early rainy season (March–May) (Fig. 5.16 and 5.17).

Table 5. 6 Numbers of births given by 19 focal females which were categorized into five groups based on their morphological and genetic characteristics (shown Table 5.1 and Figure 5.4) in 2012.

	March	April	May	Total
L_4R_0	3	2		**5**
L_3R_1	1	1		**2**
L_2R_2		4*	1	**5**
L_1R_3	2	1	1	**4**
L_0R_4		4		**4**
Total	**6 (30%)**	**12 (60%)**	**2 (10%)**	**20 (100%)**

Note: *A female of group L_2R_2 gave birth to twin infants in April 2012.

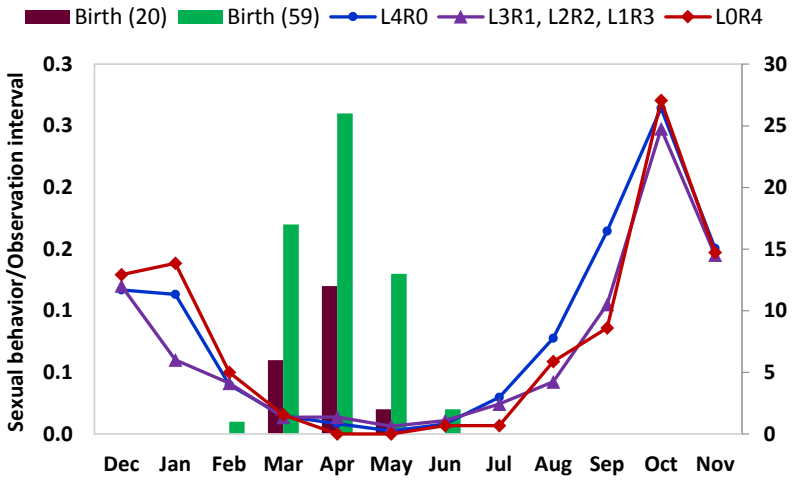

Figure 5. 16 Average rate of receptivity of all the 19 focal females in each month with the distribution of 20 births given by the 19 focal females and 59 births given by the total of 60 adult females in the same group in 2012.

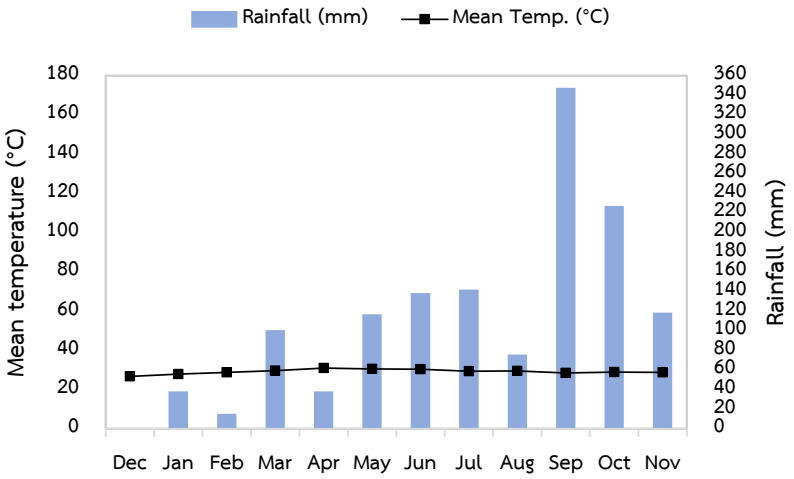

Figure 5. 17 Average monthly temperature (°C) and monthly rainfall (mm) of Chonburi Province Station from December 2011 to November 2012. (Source: Thai Meteorological Department).

Discussion

Macaques in KKZ with long-tailed-like (L_4R_0; G/G), rhesus-like (L_0R_4; A/A), and hybrid (L_3R_1, L_2R_2, L_1R_3; A/G) characteristics showed similar patterns of breeding season (Fig. 5.15), although some inter-individual differences were found (Figs. 5.5–5.14, Table 5.3). The mating season was in September–December with the most frequent successful copulations (R) in October. Their birth season was from February to June with a peak during March–May 2012 (Table 5.6, Fig. 5.16). Mating or copulation (R) was not found in rhesus-like macaques for 2–5 months during March–July in all the four focal animals (Fig. 5.13). This correlated with the birth season in March–June (Figs. 5.13–5.14, Table 5.6). Copulations of the other groups (long tailed-like L_4R_0 and hybrids L_3R_1, L_2R_2 and L_1R_3) were also rarely seen during the birth season, but one individual in L_1R_3 group copulated throughout 12 months of the year (Fig. 5.11).

The breeding season of KKZ rhesus-like macaques was comparatively resemble to that of Chinese rhesus macaques in Chongqing (29°N), Yunnan (22°N), and Hainan (18°N) whose breeding or reproductive season was from October–February (Du et al., 2010), September–January, and November–March, respectively (Fooden, 1995). One population of rhesus macaques living in Loei Province (17°14′N), Thailand was also

reported the breeding period from November to July (Malaivijitnond and Varavudhi, 2002).

Interestingly, the breeding pattern of KKZ long-tailed like macaques was different from those inhabiting Peninsular Malaysia (Kavanagh and Laursen, 1984) and Sumatra, Indonesia (van Noordwijk and van Schaik, 1985) or Sundaic long-tailed macaques. Sundaic female long-tailed macaques were pregnant all year round with a birth peak during May–July. Although the breeding and birth pattern of long-tailed like macaques were different from those Sundaic ones, they tended to be similar to Thai long-tailed macaques (14–16°N) of which the birth period was from March to May (Fooden, 1971).

The results from this study might be supported by the review of Lancaster and Lee (1965) that rhesus macaques which distribute in higher latitude (15–35°N) or temperate zone tended to have clear seasonality when comparing to long-tailed macaques who live in lower latitude around the equator (20°N–10°S) or tropical/sub-tropical zone.

In a number of Cercopithecidae, time intervals between the median or modal month of the birth peak and first month with peak food availability ranged 2–5 months (van Noordwijk and van Schaik, 1985). For rhesus macaques, it was about 4 months (Southwick et al., 1965). In this study, birth peak of KKZ (March–May) coincided with peak of fruiting period during mid-rainy season (June–August) of KKZ forest (Jadejaroen, J. personal observation) and vicinity (Khao Kaset or Khao Namsap, 13°07′N, 100°55′E) (Jadejaroen et al., 2010).

Thailand encompasses the southernmost habitat range of rhesus macaques and the northernmost habitat range of long-tailed macaques where photoperiods, rainfall, temperature, vegetation and other environmental factors are similar. It is possible that the southern or Indochinese rhesus macaques have similar breeding and birth pattern to that of northern or Indochinese long-tailed macaques. However, birth peak can vary annually according to female's condition which depends on availability of food supply, climatic condition, and her reproductive history (van Noordwijk and van Schaik, 1985).

Other than several factors mentioned previously, genetics should also play an important role in sexual behavior and breeding pattern and birth in macaques. KKZ

long-tailed macaques which were identified as Indochinese ones should have genetic admixtures of Chinese rhesus macaques by male-driven unidirectional gene flow (Tosi et al., 2002; 2003; Kanthaswamy et al., 2008; Bonhomme et al., 2009; Stevison and Kohn, 2009). Thus, this causes the breeding and birth patterns of Indochinese long-tailed macaques, including KKZ population, resemble to those of Chinese rhesus macaques, while the Sundaic long-tailed macaques, including Malaysian and Indonesian ones are evidently non-season breeders (Kavanagh and Laursen, 1984; van Noordwijk and van Schaik, 1985). From these results, it indicates that breeding pattern of seasonal breeders of rhesus macaques was dominant over that of long-tailed macaques. Thus the breeding patterns of Indochinese long-tailed macaques which carried the admixture genetic characteristics of rhesus macaques tends to be the seasonal breeders. It is not surprising to see the seasonal breeding pattern in the hybrids between the two species at KKZ. It is interesting to investigate and compare further for the breeding pattern of pureblood Chinese rhesus, Sundaic long-tailed macaques and other populations of Indochinese long-tailed macaques, especially at the natural hybrid zone (15–20°N) in the future.

CHAPTER VI
CONCLUSION AND RECOMMENDATION

Conclusion

In regard to the results of morphological and genetics characteristics, and sexual behaviors of macaques as described in Table 6.1, they supported that these macaques were hybrids between rhesus and long-tailed macaques.

Morphological characteristics of macaques from KKZ were analyzed using photogrammetry. Crown-rump length (CRL) and tail length were measured by ImageJ software (Schneider et al., 2012), and the %RTL was calculated by (Tail length/CRL) x100. RTL of > 95.6% was long-tailed macaque specific and < 70% was rhesus macaque specific. Pelage color was measured using the L*a*b* color system in Adobe Photoshop CS version 8.0 (San Jose, CA, USA), and the contrast of the yellowish pelage values (Cb*) which were calculated by the subtraction of the b* values at the waist and the back (b*waist-b*back) is a species specific of Indochinese rhesus macaques. Crown hair with the dark patch (with irregular crest) was determined as of long-tailed macaques, while no dark patch, smooth and directed posteriorly was of rhesus macaques. Transzygomatic and infrazygomatic cheek hair were determined as of long-tailed and rhesus macaques, respectively. Smaller, kite-shaped reddening of sex skin within the ischial callosity region and large upside-down u-shape reddening covers outside ischial callosity region are identified as of long-tailed and rhesus macaques, respectively. The morphological characteristics (18 character states) were scored and grouped by Multiple Correspondence Analysis (MCA) and clustered by agglomerative hierarchical cluster analysis (AHCA) in XLSTAT (Addinsoft SARL, Paris, France). KKZ macaques were categorized into five groups of L_4R_0, L_3R_1, L_2R_2, L_1R_3, and L_0R_4. . Photogrammetry, however, could be applied for the study of hybrids with intermediate characteristics.

Genetics of rhesus and long-tailed macaques were discriminated using a STAT6 SNP marker (Barr et al., 2011) and PCR-RFLP technique. The 745 bp of STAT6 gene covering the bp 491 was amplified, digested with *Apa*I and agarose gel electrophoresed. In regard to the G/G genotype of long-tailed macaque, A/A genotype

of rhesus macaque and A/G genotype of the hybrid, the two (234 and 511bp), one (745 bp) and three (234, 511 and 745bp) band patterns were received (Table 6.1). Of 118 KKZ samples, 6 (5%), 56 (47%), and 56 (47%) showed A/A (rhesus), A/G (hybrid), and G/G (long-tailed) genotype, respectively. Comparing genotypes of 31 KKZ samples, 26 (83.87%) were congruent with their morphological characteristics. The frequency of alleles A (rhesus) and G (long-tailed) from 2006 to 2014 decreased from 0.40 to 0.21 and increased from 0.06 to 0.79, respectively. Although, hybrids of post F1 generation can have A/A, A/G, or G/G genotypes, detection of some samples elsewhere with A/G genotype using STAT6 SNP PCR-RFLP technique can suggest the hybridization between rhesus and long-tailed macaques of that population.

Sexual behaviors were followed in 19 focal adult females which were chosen according to their morphological and genetic characters and belonged to one of the five morphological groups categorized above. Sexual behaviors were classified into proceptivity, attractivity, and receptivity with detailed description in an ethogram. Scan sampling method was used. Animals were followed from dawn to dust for 6–8 days/month from December 2011 to November 2012. All five monkey groups including hybrids between the two species had comparable patterns of sexual behaviors throughout the year. The patterns depicts breeding season with birth peak during March and May, especially in rhesus macaques of which the receptivity was not seen during April and May. The results gained from this study are incongruent with previous reports that rhesus macaques are strict seasonal breeder and long-tailed macaques are non-seasonal breeders. Whether this is the consequences of hybridization of these two species in the past or it is unique characters of Indochinese long-tailed macaques needs to be investigated further. The results also suggested that sexual behavior was not a marker to discriminate the hybrid between the two species living in the Indochinese region.

Morphological and genetic characteristics together with sexual behavior of rhesus, long-tailed and hybrid macaques in KKZ may represent the natural hybridization of these two species in their postulated natural hybrid zone. From approximately 20 years ago when rhesus macaques were released into the habitat of long-tailed macaques in KKZ, their fertile hybrid offspring could be detected as they

showed morphological characteristics (%RTL, Cb*, crown hair, cheek hair, and sex skin reddening) ranged intermediately between those of their parental species. All the three genotypes; A/A of rhesus, A/G of hybrid, and G/G of long-tailed macaques were found. However, from 2006 to 2014, morphological characteristics of individuals of this group seemed to be diluted as the younger generations showed more long-tailed like patterns. This was also confirmed by the recurrence of the A rhesus allele to the G long-tailed allele at the STAT6 locus.

Similarly to hybridization in their natural hybrid zone by a unidirectional gene flow of male driven of rhesus to long-tailed macaque population, a small group of rhesus macaques had recently released into the habitat of feral long-tailed macaques in KKZ and produced hybrids. While characteristics of hybrid offspring of rhesus and long-tailed macaques in KKZ were temporally changed and showed a recurrence to long-tailed macaques, in the natural hybrid zone where the hybridization occurred a million years ago the hybrids also showed a recurrence to long-tailed macaques but spatially. The hybrid zone was in a broad overlap area approximately 2,000 km stretching from southeastern Bangladesh through north and northeastern Thailand to central Vietnam downward the habitat range of long-tailed macaques reaching to the Isthmus of Kra. Although there has no previous report that depicts all the morphological, genetic and behavioral characteristics of hybrids between these two species, this study has shown interesting information of the link between morphological characteristics with their genetic characteristics and sexual behaviors.

Recommendation

1. Photogrammetry is a non-invasive technique that can be applied for studying hybrids between rhesus and long-tailed macaques. Taking several photographs of the same individuals in relatively moderate light condition and shining evenly onto the animals are suggested.

2. Collecting DNA of rectal cells from animals' fecal samples is another non-invasive method but the samples collected should be fresh and should be collected from known individuals for the prevention of repetitive sampling. Discrimination of hybrids

between rhesus and long-tailed macaques using STAT6 SNP PCR-RFLP is relatively simple, efficient, and inexpensive. Having information on genetics together with morphological characteristics of those individuals could ensure the assessment of rhesus, long-tailed and hybrid macaques.

3. The study of sexual behaviors and reproductive seasonality suggested long-tailed macaques of KKZ are seasonal breeders. This is out of the expectation when comparing with the previously reported on other populations of long-tailed macaques in Sundaic region. Thus, studying sexual behaviors and birth patterns of other Indochinese long-tailed populations should be performed.

4. For more information and knowledge on hybrids between rhesus and long-tailed macaques, continuous and long-term studies on morphological, genetic and behavioral aspects should be needed.

Table 6. 1 Morpholigical, genetic, and sexual behavioral characteristics of rhesus-like, long-tailed like, and hybrid macaques in Khao Khieow Open Zoo.

Characteristics	Rhesus-like	Hybrid	Long-tailed like
Morphology			
RTL(%)	< 69.6	< 69.6 – > 95.6	> 95.6
Cb*	> 2.01	> 2.01 – < 1.33	< 1.33
Crown hair	No dark patch, hairs smooth, directed posteriorly	Mixed, or rhesus- or long-tailed like	Dark patch in the miiddle of the head, sometimes with crest
Ckeek hair	Infrazygomatic	Mixed, or rhesus- or long-tailed like	Tranzygomatic
Sex skin reddening	Red color down to the inguinal part. Usually had a kite shape and swelling at the base of the tail. Reddening rim was usually covered narrower area and within the ischial callosity area.	Rhesus- or long-tailed like	Red color down to the thigh, and usually had an up-side down U shape. Reddening rim was covered larger area and out of the ischial callosity area.
Genetics			
STAT6 genotype	A/A	A/G (AA, GG)	G/G
Sexual behavior			
Seasonal/Non-seasonal breeder	Seasonal breeder	Seasonal breeder	Seasonal breeder
Breeding season	September–January, no copulation in April–May	September–January	September–January
Birth season	February–June	February–June	February–June
Birth peak	March–May	March–May	March–May

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2. Jadejaroen J, Kawamoto Y, Hamada Y, Malaivijitnond S. (in press). A SNP marker at the STAT6 locus can identify the hybrids between rhesus (Macaca mulatta) and long-tailed macaques (M. fascicularis) in Thailand: a rapid and simple screening method and its application. Primates, xx, xx–xx. DOI: 10.1007/s10329-015-0502-2.