Anthropogenic Macaque Hybridization and Genetic Pollution of a Threatened Population

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ABSTRACT.– Interspecific matings between one released male pig-tailed macaque and female rhesus macaques were observed in a small isolated semi-wild troop of rhesus macaques in northeastern Thailand. Two of three juvenile males suspected to be hybrids based on their appearance, were caught and examined morphologically and genetically. Both suspected hybrids had a dark brown and anteriorly narrow crown patch and thinly haired tails as are common in pig-tailed macaques, and tail-lengths in the range of rhesus macaque. Male no. 19 showed neither cheek hair whorl nor the bipartite pattern of pelage color characteristics of rhesus macaque, whereas male no. 14 displayed both of these characters. We could not determine whether they were truly hybrids based on our morphological assessments and so both animals were also examined genetically. Study of variation at the Y-chromosome linked TSPY locus showed that although monkey no. 19 was sired by the pig-tailed macaque, monkey no. 14 was sired by a rhesus macaque. A mtDNA analysis indicated that both suspected hybrids were sons of rhesus mothers. Electrophoretic examination of blood hemoglobin-α protein confirmed that only monkey no. 19 was truly a hybrid. Although only one individual was confirmed to be hybrid in this troop, the hybridization could become a severe threat to the genetic integrity of the native troop and may hinder the tracing of the evolutionary history of the population. It is especially true in the rhesus populations which are now very rare in Thailand.

KEY WORDS: hybridization; *Macaca nemestrina*; *Macaca mulatta*; bipartite pelage-color; TSPY gene

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INTRODUCTION

Rhesus macaques (*Macaca mulatta*) occurred historically across northern Thailand from Uthai Thani Province in the

west, to Nan and Chiang Mai Provinces in the center, and Nongkhai and Loei Provinces in the northeast (Fooden, 2000; Malaivijitnond et al., 2005). Now rare, their range has been greatly reduced by rapid and extensive habitat deterioration by logging, deforestation and agricultural development. In addition to the habitat loss and fragmentation, the surviving wild macaque populations are threatened by more direct human activities, including pet-release and genetic pollution (Malaivijitnond et al., 2005).

We recently reported on a troop of rhesus macaques inhabiting the Wat Tham Pa Mak Ho (WTPMH), a Buddhist temple, in Loei Province, northeastern Thailand (Malaivijitnond and Varavudhi, 2002). The WTPMH troop is the first new population of Thai rhesus macaques reported since the 1960's. In 2000 or a few years after our first census, one pet male pig-tailed macaque (*M. nemestrina*) was released into this troop. As the WTPMH troop is spatially isolated from other macaque troops by vast agricultural fields, the male pig-tailed macaque was forced to join the WTPMH's rhesus troop. In nature, rhesus macaques are reproductively isolated from pig-tailed macaques by behavioral and ecological differences (Fooden, 1982), and do not normally interbreed with each other in the wild. However, being twice as heavy as adult male rhesus macaques, a pig-tailed macaque at WTPMH became the alpha male and acquired access to reproductively active female rhesus macaques (Malaivijitnond and Varavudhi, 2002; Hamada et al., 2006). In March 2003, three immature males were found to have mixed morphological characters of the two species and were suspected to be hybrids (Hamada et al., 2006). But, as it is difficult to judge

hybrid status based solely on morphology, we also determined their characteristics using maternal and paternal molecular genetic markers.

MATERIALS AND METHODS

Subject population

The population of rhesus macaques inhabiting the Wat Tham Pa Mak Ho in Wang Sapung District, Loei Province, northeastern Thailand (GPS 17° 14′ 56″N 108° 39′ 50″E) was studied. The temple is located on a limestone hill (340 m above sea level) covered mainly with spiny shrubs, bamboo and fig trees and surrounded by fields of rice, soybean, cassava, banana and tamarind. One-hundred and nine rhesus macaques were counted in March, 2003, including 41 adults (6 males and 35 females), 12 sub-adults, 38 juveniles and 18 infants. The WTPMH troop was composed of one main troop, one male group, and two solitary males. A released pig-tailed macaque was found to be an alpha male of the main troop. During breeding season in March 2003, he was observed to mate with three female rhesus macaques.

Animal capture and field work

In March 2003, 33 macaques (11 males and 22 females), including a pig-tailed macaque and two of the three suspected hybrids (monkey nos.14 and 19), were temporarily caught with a net trap (W x L x H = 6 x 6 x 2 m). Monkeys were anesthetized by intramuscular injection with 10 mg/kg body-weight of ketamine hydrochloride. While the monkeys were immobilized, body mass, tail length, crown-rump length, and pelage color were recorded, blood samples were collected,

and overall health was examined. The monkeys were released back to the troop after their complete recovery from anesthesia. The ages of monkeys were estimated from the dental eruption according to Smith et al. (1994).

Determination of relative tail length

The crown-rump length and tail length were measured with an anthropometer (Iwamoto, 1971). A relative tail length (%) was obtained by the division of the tail length by the crown-rump length.

Determination of pelage color

Pelage color was quantitatively determined using a color reflectometer (Color Analyzer™ Model CR-200, Minolta Co., Ltd, Japan) following the method described by Hamada et al. (2005; 2006). Color is expressed by three parameters: Lightness, ranging from dark (0) to light (100); hue of green (-60) to red (+60); and hue of blue (-60) to yellow (+60). We measured color at the vertex of the head, the back (interscapular), lateral upper arm and forearm, dorsum of the hand, waist (suprailiac), lateral thigh and leg, and dorsum of the foot.

Blood collection

Blood samples (3 ml/kg BW, maximum 10 ml) were collected from the monkeys by femoral venipuncture and immediately mixed with 500 i.u. of heparin (Leo Pharmaceutical Products, Denmark). Blood plasma, a buffy-coat, and red blood cells were separated by centrifugation at 1,000 xg for 10 minutes. DNA was extracted from the buffy-coat following the method described by Sambrook et al. (1989) and used for mtDNA and TSPY gene analysis.

PCR primers for amplification and sequence determination

Mitochondrial DNA

Mitochondrial DNA was analyzed to confirm the maternal lineage of the two suspected hybrids (Hayasaka et al., 1991; Evans et al., 2001). A portion of the mitochondrial genome spanning the control region (or D-loop region) in both suspected hybrids and 14 selected WTPMH rhesus macaques was amplified and sequenced. Primers used for the PCR amplification followed Hayasaka et al. (1991), including saru-4: 5'-ATC AGG GTC TAT CAC CC TAT-3' and saru-5: 5'-GGC CAG GAC CAA GCC TAT TTG-3'. Another couple of sequencing primers was designed from the mtDNA sequence of Japanese macaque (*M. fuscata*) as follows; 554F: 5'-ATC ACC CTA TTT AAC CAG TCA C-3' and 567F: 5'-AAA CCC ATC TAG GCA TTT TCA G-3'. The 5' end of the primer 554F and the 3' end of the primer 567F correspond to the nucleotide number 18 and 831 of the Japanese macaque MNR sequence (Hayasaka et al. 1991), respectively. The primer saru-4 was biotinylated, whereas the primers 554F and 567F were FITC labeled (Japan Bioservice, Japan). The PCR amplification of the mtDNA gene was achieved using AmpliTaq Gold™ kit (Applied Biosystems, Japan). The amplification was carried out in a Perkin-Elmer Cetus Thermal Cycler (model 9600) for 30 cycles consisting of denaturation at 94ºC for 1 min, annealing at 52ºC for 1 min, and extension at 72ºC for 2 min. The whole 30 PCR cycles were preceded by an initial denaturation step of 94ºC for 10 min and followed by a final extension period of 72ºC for 10 min. PCR products were purified by Microcon YM-100 Centrifugal

Filter Devices (Millipore Corporation, USA).

Sequencing of the PCR products was performed as in a single-stranded DNA (ssDNA) form using a Thermo Sequenase Cycle Sequencing Kit (USB Corporation, USA). The ssDNA was prepared using streptavidin magnetic beads in combination with a biotinylated primer followed Malaivijitnond et al. (2002). The nucleotide sequence was determined for both directions using a fluorescent automated sequencer (DSQ-1, Shimadzu, Japan).

Mitochondrial DNA sequences of rhesus macaques from India and China reared at the Primate Research Institute of Kyoto University in Japan (PRI) were also analyzed and used as in-group references for phylogenetic analysis. As we could not sequence the mtDNA of the pig-tailed macaque, the comparable sequence from the Barbary macaque (*M. sylvanus*; Arnason et al., 2000), a closely related macaque species (Morales and Melnick, 1998; Tosi et al., 2000; 2003; Deinard and Smith, 2001), was used as an out-group reference for mtDNA analysis.

TSPY gene

Since both of the suspected hybrids were males, they were assumed to have inherited their Y-chromosome from the male pig-tailed macaque. The TSPY (testis-specific protein on the Y chromosome) gene was selected for this study, because it is evolutionarily conserved and shows species-specificity in macaques (Tosi et al., 2000). The 711-bp TSPY gene sequences studied included parts of exon 1, exon 2 and the first intron, was amplified using the following primers: TSPY-A: 5′-AGC CAG GAA GGC CTT TTC TCG-3′ and TSPY-B: 5′-CCA TGT AGC TCA GCA TGT

CTT CAT-3′ (Kim and Takenaka, 1996). These partial TSPY gene sequences were analyzed in one male pig-tailed macaque and ten male rhesus macaques (including suspected hybrid nos. 14 and 19). The 5′ of primer TSPY-A and the 3′ of primer TSPY-B respectively correspond to nucleotide number 324 and 1093 of the human sequence (Zhang et al., 1992). Amplification of the TSPY gene was carried out in a Perkin-Elmer Cetus Thermal Cycler (model 9600) for 30 cycles consisting of denaturation at 94ºC for 1 min, annealing at 60ºC for 1 min, and extension at 72ºC for 2 mins using the AmpliTaq Gold™ Kit (Applied Biosystems, Japan). The main PCR amplification cycle was preceded by an initial denaturation step of 94ºC for 10 min and followed by an additional extension period of 72ºC for 10 min. The PCR product was electrophoresed through 2% Nusieve^R GTG^R Agarose gel electrophoresis (Rockland, USA) and the expected fragment was recovered from the gel using glass powder (Easytrap™ Ver.2, Takara Bio Inc, Japan). The TSPY gene PCR product was directly sequenced using a Thermo Sequenase Cycle Sequencing Kit (USB Corporation, USA). The primer for sequencing was designed in our laboratory from the macaque TSPY gene sequences (Tosi et al., 2000) as follows: TSPY-SEQ: 5′-GGA GGG AGA GGA GAA AGG GA-3′ and TSPY-SEQR: 5′-TCC CTT TCT CCT CTC CCT CC-3′. The 5′ of primer TSPY-SEQ and the 3′ of primer TSPY-SEQR correspond to nucleotide number 546 of the rhesus sequence and number 548 of the pig-tailed sequence, respectively. The primers TSPY-SEQ and TSPY-SEQR were FITC labeled (Japan Bioservice, Japan). A nucleotide sequence of TSPY gene was determined using a fluorescent

FIGURE 1. Morphological characters of released male pig-tailed macaque (A), an adult female rhesus macaque (B), and the hybrid no. 19 (C). The dark brown crown patch and a slender, thinly-furred and shorter tail are found in pig-tailed macaques. The bipartite pattern of pelage color and the fluffy and longer tail clearly characterize the rhesus macaque. The hybrid shows mixed characters of those two species.

automatic sequencer (DSQ-1, Shimadzu). TSPY gene sequences of Indian and Chinese rhesus macaques and Thai pig-tailed macaque were obtained from Tosi et al. (2000) and used as in-group references, and a TSPY gene sequence of Thai stump-tailed macaques (*M. arctoides*; Tosi et al., 2000) was used as an out-group reference for phylogenetic analysis.

Analyses of sequence data

The nucleotide sequences were edited manually with the GENETYX program (version 5.0, SDC Tokyo, Japan) and transformed into a sequence-data matrix using the ClustalX multiple alignment program. Phylogenetic trees were constructed from the genetic distance data (Kimura's two parameters) based on the neighbor-joining method (Saitou and Nei, 1987) using the MEGA program (version 3). Robustness of the tree was assessed with branch supporting-values from bootstrap (BS) statistic analyses (1,000 replicates).

Electrophoretic analysis of blood hemoglobin protein

Hemoglobin protein from the red blood cells of a male pig-tailed macaque, a male WTPMH rhesus macaque, and the two suspected hybrids was analyzed as described previously by Nozawa et al. (1977). Electrophoretic patterns of hemoglobin of male rhesus macaques from India and China (reared at the PRI), and one male and one females pig-tailed macaques from Nan Province, northern Thailand, were also determined for comparative purposes.

RESULTS

Pelage character and relative tail-length

The male pig-tailed macaque at WTPMH had golden brown-agouti hairs around the mid-dorsal region of the trunk with lighter color on the underparts and a dark brown crown patch (Figs. 1A and 2). Its slender and thinly-furred tail arched forward over its back and the relative tail-length of 36.6% of crown-rump length (Fig. 1A). From these characteristics, this individual is considered to be *M. nemestrina leonina* (Fooden, 1975). Rhesus macaques in WTPMH had a pelage color
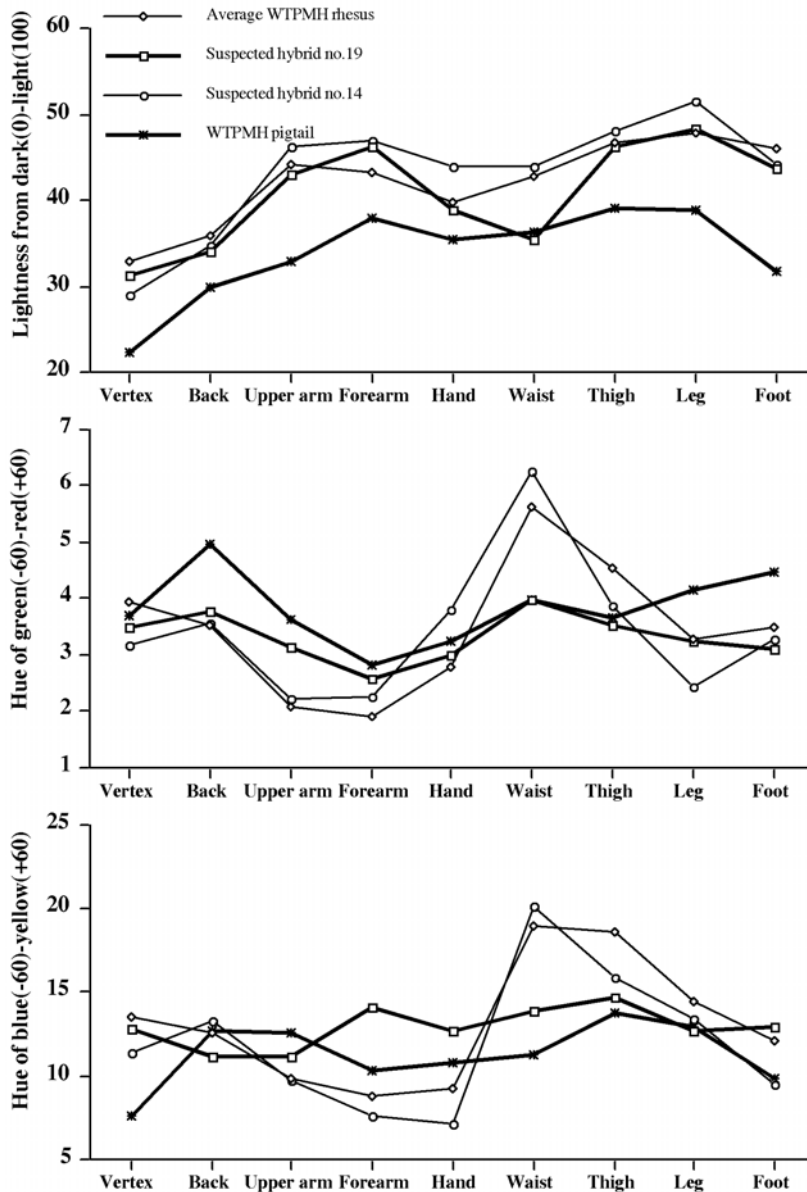
FIGURE 2. Pelage colors in WTPMH rhesus macaques, the WTPMH pig-tailed macaque and the suspected hybrids.

of a bipartite pattern, which is a key species-specific character for *M.mulatta* (Fooden, 2000). That is, the fur of the lower back is conspicuously lighter and more reddish and yellowish than that of the upper back (Figs. 1B and 2). The pelage of rhesus macaques was lighter than that of

the pig-tailed macaque (Fig. 2). Their crown hairs are pointed smoothly posterior, and cheek hairs usually form a small crest or whorl at the cheek. The facial skin is thinly haired and reddish. The tail is fluffy and particularly longer $(57.0 \pm 4.2\%,$ S.D.) than that of the pig-tailed macaque. The
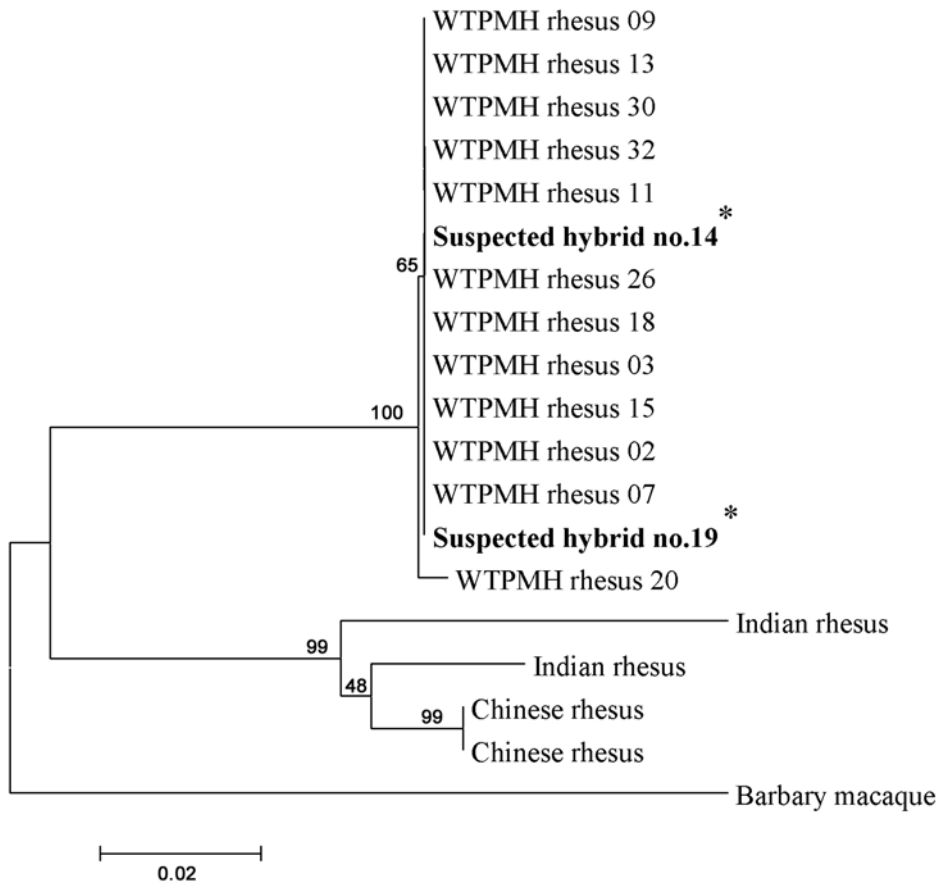
FIGURE 3. The phylogenetic tree constructed from 454-bp of mitochondrial DNA sequences. Values along branches represent the percentages of times that these branches occurred in 1,000 times generated by bootstrap analyses of the original data.

body mass of the pig-tailed macaque was 13.1 kg, whereas the heaviest adult male rhesus macaque was only 6.45 kg.

The three suspected hybrids were seen to have a dark brown and anteriorly narrow crown patch and thinly haired tails as are common in pig-tailed macaques (Fig. 1C). Their tails, however, were long and appeared to be within the range of rhesus macaques. Two of the three suspected hybrids (nos. 14 and 19) were caught with a net trap. The estimated ages of no. 14 and no. 19 were 1 and 3 years, respectively. They had relative tail lengths of 65.5 and 54.2%, respectively. Although

the tail of no. 19 was relatively longer than that of adult pig-tailed macaque, its tail was the shortest in this age cohort (1 – 4 years old). Animal no. 19 showed neither hair whorl at the cheek nor the bipartite pattern of rhesus macaques, whereas animal no. 14 displayed both the bipartite pattern of pelage color and the whorl of hair at the cheek (Fig. 1). Based solely on the above mentioned morphological characters, it is not possible to determine the maternal and paternal lineages of the two suspected hybrids. The third putative hybrid escaped genetic investigation at this time and is not considered further here.
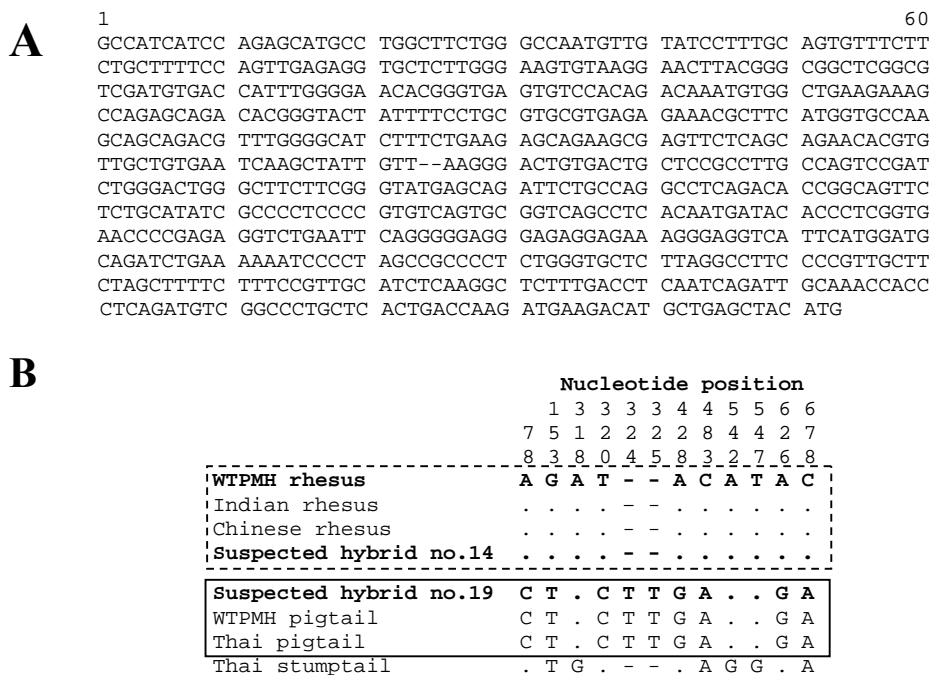
A

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1                                                           60
GCCATCATCC AGAGCATGCC TGGCTTCTGG GCCAATGTTG TATCCTTTGC AGTGTTTCTT
CTGCTTTTCC AGTTGAGAGG TGCTCTTGGG AAGTGTAAGG AACTTACGGG CGGCTCGGCG
TCGATGTGAC CATTTGGGGA ACACGGGTGA GTGTCCACAG ACAAATGTGG CTGAAGAAAG
CCAGAGCAGA CACGGGTACT ATTTTCCTGC GTGCGTGAGA GAAACGCTTC ATGGTGCCAA
GCAGCAGACG TTTGGGGCAT CTTTCTGAAG AGCAGAAGCG AGTTCTCAGC AGAACACGTG
TTGCTGTGAA TCAAGCTATT GTT--AAGGG ACTGTGACTG CTCCGCCTTG CCAGTCCGAT
CTGGGACTGG GCTTCTTCGG GTATGAGCAG ATTCTGCCAG GCCTCAGACA CCGGCAGTTC
TCTGCATATC GCCCCTCCCC GTGTCAGTGC GGTCAGCCTC ACAATGATAC ACCCTCGGTG
AACCCCGAGA GGTCTGAATT CAGGGGGAGG GAGAGGAGAA AGGGAGGTCA TTCATGGATG
CAGATCTGAA AAAATCCCCT AGCCGCCCCT CTGGGTGCTC TTAGGCCTTC CCCGTTGCTT
CTAGCTTTTC TTTCCGTTGC ATCTCAAGGC TCTTTGACCT CAATCAGATT GCAAACCACC
CTCAGATGTC GGCCCTGCTC ACTGACCAAG ATGAAGACAT GCTGAGCTAC ATG
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B

	Nucleotide position									
	1	3	3	3	3	4	4	5	6	6
	7	5	1	2	2	2	8	4	4	2	7
	8	3	8	0	4	5	8	3	2	7	6	8
WTPMH rhesus	A	G	A	T	-	-	A	C	A	T	A	C
Indian rhesus	.	.	.	.	-	-	.	.	.	.	.	.
Chinese rhesus	.	.	.	.	-	-	.	.	.	.	.	.
Suspected hybrid no.14	.	.	.	.	-	-	.	.	.	.	.	.
Suspected hybrid no.19	C	T	.	C	T	T	G	A	.	.	G	A
WTPMH pigtail	C	T	.	C	T	T	G	A	.	.	G	A
Thai pigtail	C	T	.	C	T	T	G	A	.	.	G	A
Thai stumptail	.	T	G	.	-	-	.	A	G	G	.	A

FIGURE 4. Nucleotide sequences for the studied portion of the TSPY gene in WTPMH rhesus macaques (A), and the comparison with those of the Indian and Chinese rhesus macaques, suspected hybrids, WTPMH pig-tailed macaque and reported sequences of Thai pig-tailed and Thai stump-tailed macaques (Tosi et al. 2000). Dashes (-) indicate gaps.

Maternal lineage of suspected hybrids resolved by mtDNA sequence analysis

A 454-bp portion of the mtDNA spanning the control region in rhesus macaques from WTPMH, India and China was sequenced and compared. The phylogenetic tree shows that these three groups of rhesus macaques are monophyletically separated from the Barbary macaque (Arnason et al., 2000) (Fig. 3). Within the rhesus macaques two clusters are evident: one involving the WTPMH macaques and the other the macaques from India and China. The WTPMH macaques cluster into a single haplotype. The two suspected hybrids (nos. 14 and 19) lie within the WTPMH rhesus macaque cluster. This result is interpreted as evidence that the two putative hybrids were sons of the WTPMH rhesus females.

Paternal lineage of suspected hybrids resolved by TSPY gene

The eight WTPMH rhesus macaques were identical at the 711-bp TSPY sequence studied. These monkeys were also identical to those of the Indian and Chinese rhesus macaques previously reported by Tosi et al. (2000) (Fig. 4A, B). Further-more, 100% similarity of 713-bp of TSPY gene sequence was also observed between the WTPMH pig-tailed macaque and conspecifics from Thailand (Tosi et al., 2000) (Fig. 4B). Compared with the sequence of rhesus macaques, the TSPY gene sequences of pig-tailed macaques have seven different nucleotide positions (numbers 78, 153, 320, 428, 483, 626, and 678) and two successive nucleotide insertions (at positions 324 and 325) (Fig. 4B). This interspecific difference is comparable to those between rhesus and
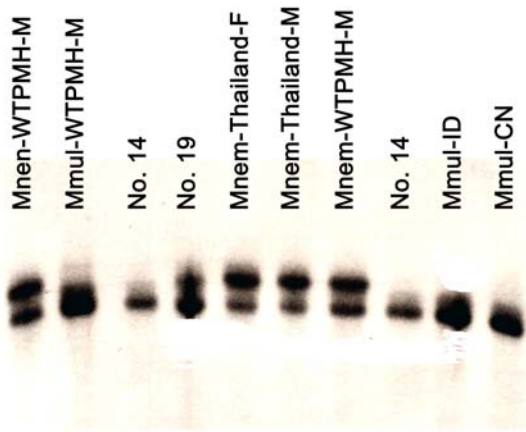
FIGURE 5. The electrophoretic patterns of hemoglobin-α protein. Mnem = *Macaca nemestrina*, Mmul = *M. mulatta*, F = female, M = male, and Nos. 14 and 19 = the suspected hybrid nos. 14 and 19.

stump-tailed macaques (six different nucleotide positions) or between pig-tailed and stump-tailed macaques (seven different nucleotide positions and two successive nucleotide insertions).

The paternally inherited TSPY gene sequence of suspected hybrid no. 14 showed a 100% similarity with those of rhesus macaques, whereas suspected hybrid no. 19 showed a 100% similarity with those of the two pig-tailed macaques (Fig. 4B).

Parental lineage of suspected hybrids resolved using blood hemoglobin protein

The electrophoretic examination of blood hemoglobin protein clearly showed that the electrophoretic patterns of rhesus macaques are different from those of pig-tailed macaques (Fig. 5). Only one band of the electrophoretic patterns was observed in all rhesus macaques from various origins: WTPMH, India and China. However, two bands were found in all pig-tailed macaques from Thailand (inhabiting the WTPMH and Nan Province). In agreement with the TSPY gene result, the suspected hybrid no.

19 had two bands, while the suspected hybrid no. 14 had only one band. Moreover, the intensity of the electrophoretic bands of monkey no. 19 was different from those of pig-tailed macaques, that is, the faster band was higher intensity than the slower band.

DISCUSSION

In Thailand, the hybridization of different macaque species caused by human activities has become a serious problem for the conservation of the remaining wild and free-ranging commensal populations (Hamada et al., 2004; Malaivijitnond et al., 2005). In many Thai localities, released pet macaques are found in free-ranging troops of the same or different species. For example, one released female stump-tailed macaque (*M. arctoides*) and two female pig-tailed macaques were found in a long-tailed (*M. fascicularis*) troop in Lopburi Province, central Thailand, and another released female long-tailed macaque was found in a stump-tailed troop in Petchaburi Province, upper peninsular Thailand (Hamada et al., 2004; Malaivijitnond et al., 2005). Such releases could result in interspecific hybridization, as found in the WTPMH. Such interspecific hybridization will spoil the genetic integrity of the native troop, and hinder attempts to trace the evolutionary history of the population.

Based solely on the morphological characters of the three suspected hybrids found in the WTPMH troop, it was not possible to determine their maternal and paternal lineages, especially as these were immature individuals. Genetic analysis on appropriate marker genes was shown to be necessary. We chose the mtDNA and TSPY gene to trace back the maternity and

paternity of those hybrids. The mtDNA sequence used in this study is a hypervariable segment II (HVS II) region as previously reported in Assamese macaques (*M. assamensis*) and Japanese macaques (*M. fuscata*) (Kawamoto et al., 2006; 2007), which is different from a hypervariable segment I (HVS I) region (Smith and McDonough, 2005; Kyes et al., 2006) reported in Chinese and Indian rhesus macaques. However, the results of phylogenetic analysis by these two regions are found to be congruent with each other by our analysis (data not shown). The mtDNA sequence analysis showed conclusively that the two suspected hybrids that we sampled were the sons of local rhesus mothers. However, based on the TSPY sequence analysis, only the suspected hybrid no. 19 was a true hybrid sired by the released male pig-tailed macaque. The possibility that the hybrid no. 19 was sired by another pig-tailed macaque is remote, because the closest troop of pig-tailed macaques to WTPMH is approximately 300 km away (Malaivijitnond et al., 2005). Our results demonstrate that the TSPY sequence studied is very useful for tracing the paternity of macaque hybrids. As TSPY is a gene family located on the non-recombining portion of Y-chromosome (Dorit et al., 1995; Schempp et al., 1995; Affara et al., 1996) and is a highly conserved and species-specific gene (Kim and Takenaka 1996; Tosi et al., 2000) it is the genetic marker of choice to identify male interspecific hybrids

Although the uniparentally inherited non-recombining markers such as the mtDNA (control region) and nuclear TSPY gene permit the direct examination of the maternal and paternal contributions to hybridization, the screening of other genetic variation, such as allozymes or other nuclear DNA markers may provide additional information. Using the simple and inexpensive electrphoretic examination of a variable blood protein we were able to elucidate the parental lineage of suspected hybrids and confirm the results of the mtDNA and TSPY analyses. In agreement with Nozawa et al. (1977), the pig-tailed macaques from Thailand had the FS-type ($\propto^A / \propto^Q$) of Hb-$\propto$ protein with two electrophoretic bands and the rhesus macaques from India, China and Thailand had SS-type ($\propto^A$) with one electrophoretic band. However, Fooden and Lanyon (1989) reported that the pig-tailed macaques from Thailand had only 65% of major alleles of FS-type (their allele "2"). Monkey no. 19 showed two electrophoretic bands in which faster band was higher intensity than the slower one, which was reversed from those of pig-tailed macaques, whereas the monkey no. 14 had only one faster electrophoretic band. As the genetic system of Hb-$\propto$ is composed of multiple loci and some of them are linked together, this phenotypic pattern can be explained that monkey no. 19 received at least one allele of F from his pig-tailed father and one (probably multiple) allele of S from his pig-tailed father and rhesus mother, whereas the monkey no. 14 inherited only two alleles of SS-type from his rhesus parents.

Based on the DNA, blood protein and morphological analyses of hybrid no. 19, we hypothesize that the non-bipartite pattern of pelage color and the thinly-furred tail of the pig-tailed macaques are dominant over the rhesus characters, while the relative tail-length of >50% of the rhesus macaques is dominant over the pig-tailed character. The exact inheritance of these

presumably polygenic or quantitative characters needs to be further explored as additional hybrids are discovered.

Rhesus populations in Thailand are now rare and are isolated from each other by the fragmentation of their habitats. The remained populations of rhesus macaques can suffer from artificial genetic influences stemming from pet release and troop transfers. There are numerous reports on macaques where a single male temporarily associates with a troop of a different species (Bernstein and Gordon, 1980). Generally, based on mtDNA, TSPY and autosomal genes (NRAMP1, C4 intron 9 and IRBP intron 3) (Morales and Melnick, 1998; Tosi et al., 2000; 2003; Deinard and Smith, 2001), rhesus macaques and pig-tailed macaques are not sister taxa. Rhesus macaques belong to *fascicularis species group* and pig-tailed macaques belong to *silenus species group*. In nature, they are isolated by behavioral and ecological difference (Fooden, 1982). The human-made hybridization between these two species in the WTPMH will probably have significant negative consequence rather than to be creative as emphasized previously by Arnold and Meyer (2006) for wild populations. In the case of the WTPMH troop, the released pet pig-tailed macaque was forced to remain with the rhesus troop and resulting hybridization is obviously not natural. Such hybridization may make it difficult to trace the evolutionary history or phylogeography of the populations. We refer to such potentially harmful anthropogenic interspecific hybridization as "genetic pollution". Although only one hybrid was identified in the WTPMH troop in 2003, the release of pet macaques into other macaque troops should be regulated,

avoided and deterred, because the hybrid macaques can be fertile (Bernstein and Gordon, 1980). It is unknown whether first generation hybrid macaques show heterosis or hybrid vigor but numerous examples in other species show that backcrosses and later generation hybrids lose fitness through a suite of processes termed outbreeding depression, the opposite of better-known inbreeding depression. Outbreeding depression is becoming more important in small isolated populations of animals of conservation concern, especially as government policies and private initiatives result in unwanted animals being reintroduced "to the wild" to rejoin their kin. The potential significance of outbreeding depression is stressed in both of the two leading conservation genetics textbooks (Frankham et al., 2002; Allendorf and Luikart, 2007).

LITERATURE CITED

Affara, N., Bishop, C., Brown, W., Cooke, H., Davey, P., Ellis, N., Graves, J.M., Jones, M., Mitchell, M., Rappold, G., Tyler-Smith, C., Yen, P. and Lau, Y.F.C. 1996. Report of the second international workshop on Y chromosome mapping 1995. Cytogenetic and Cell Genetics, 73: 33-76.

Allendorf, F.W. and Luikart, G. 2007. Conservation and the Genetics of Populations. Blackwell, Malden, M.A.

Arnason, U., Gullberg, A., Burguete, A.S. and Janke, A. 2000. Molecular estimates of primates divergences and new hypotheses for primate dispersal and the origin of modern humans. Hereditas, 133: 217-228.

Arnold, M.L. and Meyer A. 2006. Natural hybridization in primates: One evolutionary mechanism. Zoology, 109: 261-276.

Bernstein, I.S. and Gordon, T.P. 1980. Mixed taxa introductions, hybrids and macaque systematics. In: Lindburg DG (ed). The Macaques: Studies in Ecology, Behavior and Evolution. Van Nostrand Reinhold Company, New York, pp 125-147.

Deinard, A. and Smith, D.G. 2001. Phylogenetic relationships among the macaques: evidence from the nuclear locus *NRAMP1*. Journal of Human Evolution, 41: 45-59.

Dorit, R.L., Akashi, H. and Gilbert, W. 1995. Absence of polymorphism at the ZFY locus on the human Y chromosome. Science, 268: 1183-1185.

Evans, B.J., Supriatna, J. and Melnick, D.J. 2001. Hybridization and population genetics of two macaque species in Sulawesi, Indonesia. Evolution; International Journal of Organic Evolution, 55: 1686-1702.

Fooden, J. 1975. Taxonomy and evolution of liontail and pigtail macaques (Primates: Cercopithecidae). Fieldiana Zoology, 67: 1-169.

Fooden, J. 1982. Ecogeographic segregation of macaque species. Primates, 23: 574-579.

Fooden, J. and Lanyon, S.M. 1989. Blood-protein allele frequencies and phylogenetic relationships in Macaca: a review. American Journal of Primatology, 17: 209-241.

Fooden, J. 2000. Systematic review of rhesus macaque, *Macaca mulatta* (Zimmermann, 1780). Fieldiana Zoology, 96: 1-180.

Frankham, R., Ballou, J.D. and Briscoe, D.A. 2002. Introduction to Conservation Genetics. Cambridge University Press, Cambridge.

Hamada, Y., Ohsawa, H., Urasopon, N. and Malaivijitnond, S. 2004. Preliminary report on the distribution and present status of macaques in Thailand. Primate Research, 20: 97-108.

Hamada, Y., Urasopon, N., Hadi, I. and Malaivijitnond, S. 2006. Body size, proportions and pelage color of free-ranging rhesus macaques (*Macaca mulatta*) from a zone of hybridization in northeastern Thailand. International Journal of Primatology, 27: 497-513.

Hamada, Y., Watanabe, T., Chatani, K., Hayasawa, S. and Iwamoto, M. 2005. Morphometrical comparison between Indian- and Chinese-derived rhesus macaques (*Macaca mulatta*). Anthropological Science, 113: 183-188.

Hayasaka, K., Ishida, T. and Horai, S. 1991. Heteroplasmy and polymorphism in the major noncoding region of mitochondrial DNA in Japanese monkeys: association with tandemly repeatedn sequences. Molecular Biology and Evolution, 8: 399-415.

Iwamoto, M. 1971. Morphological studies of *Macaca fuscata*: VI. Somatometry. Primates, 12: 151-174.

Kawamoto Y, Aimi M. and Sherub, T.W. 2006. Distribution of Assamese macaques (*Macaca assamensis*) in the inner Himalayan region of Bhutan and their mtDNA diversity. Primates, 47: 388-392.

Kawamoto, Y., Shotake, T., Nozawa, K., Kawamoto, S., Tomari, K., Kawai, S., et al. 2007. Postglacial population expansion of Japanese macaques (*Macaca fuscata*) inferred from mitochondrial DNA phylogeography. Primates, 48: 27-40.

Kim, H.S. and Takenaka, O. 1996. A comparison of TSPY genes from Y-chromosomal DNA of the great apes and humans: sequence, evolution, and phylogeny. American Journal of Physical Anthropology, 100: 301-309.

Kyes, R.C., Jones-Engel, L., Chalise, M.K., Engel, G., Heidrich, J. and Grant, R. 2006. Genetic characterization of rhesus macaques (*Macaca mulatta*) in Nepal. American Journal of Primatology, 68: 445-455.

Malaivijitnond, S. and Varavudhi, P. 2002. The last possible troop of semi-wild rhesus macaque (*Macaca mulatta*) in Thailand. The Natural History Journal of Chulalongkorn University, 2: 59-61.

Malaivijitnond, S., Takenaka, O., Anukulthanakorn, K. and Cherdshewasart, W. 2002. The nucleotide sequences of the parathyroid gene in primates (suborder Anthropoidea). General and Comparative Endocrinology, 125: 67-78.

Malaivijitnond, S., Hamada, Y., Varavudhi, P and Takenaka, O. 2005. The current distribution and status of macaques in Thailand. The Natural History Journal of Chulalongkorn University, Supplement 1: 35-45.

Morales, J.C. and Melnick, D.J. 1998. Phylogenetic relationships of the macaques (cercopithecidae: *Macaca*), as revealed by high resolution restriction site mapping of mitochondrial ribosomal genes. Journal of Human Evolution, 34: 1-23.

Nozawa, K., Shotake, T., Ohkura, Y. and Tanabe, Y. 1977. Genetic variations within and between species of Asian macaques. Japan Journal of Genetics, 52: 15-30.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4: 406-425.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Schempp, W., Binkele, A., Arnemann, J., Glaser, B., Ma, K., Taylor, K., Toder, R., Wolfe, J., Zeitler, S. and Chandley, A.C. 1995. Comparative mapping of YRRM- and TSPY-related cosmids in man and hominid apes. Chromosome Research, 3: 227-234.

Smith, B.H., Crummett, T.L. and Brandt, K.L. 1994. Ages of eruption of primate teeth: A compendium for aging individuals and comparing life histories. Yearbook of Physical Anthropology, 37: 177-231.

Smith, D.G. and McDonough J. 2005. Mitochondrial DNA variation in Chinese and Indian rhesus macaques (*Macaca mulatta*). American Journal of Primatology, 65: 1-25.

Tosi, A.J., Morales, J.C. and Melnick, D.J. 2000. Comparison of Y chromosome and mtDNA phylogenies leads to unique inferences of macaque evolutionary history. Molecular Phylogenetics and Evolution, 17: 133–144.

Tosi, A.J., Morales, J.C. and Melnick, D.J. 2003. Paternal, maternal, and biparenteral molecular markers provide unique windows onto the evolutionary history of macaque monkeys. Evolution, 57: 1419-1435.

Zhang, J.S., Yang-Feng, T.L., Muller, U., Mohandas, T.K., de Jong, P.J. and Lau, Y.F.C. 1992. Molecular isolation and characterization of an expressed gene from the human Y chromosome. Human Molecular Genetics, 1: 717-726.