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Chromosomal and molecular studies of a hybrid between red-shanked douc langur (*Pygathrix nemaeus*) and Hatinh langur (*Trachypithecus laotum hatinhensis*)

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Summary

Four male douc langurs (*Pygathrix nemaeus*) and a group of Hatinh langurs (*Trachypithecus laotum hatinhensis*) were released to a five hectare semi-wild enclosure at the Endangered Primate Rescue Center, Cuc Phuong National Park, Vietnam. After one month, the douc langurs approached the Hatinh langur group and copulation of a douc langur male and a Hatinh langur female was observed. After a gestation period of 205-207 days, a hybrid was born.

The coloration of the newborn hybrid more closely resembled a douc langur, but changed over a period of about four months to complete black. Some features of the newborn's appearance resembled douc langurs, such as the capped-like form of the hairs on the head, the long and grey whiskers and the tassel on the tail.

Chromosomal and molecular genetic investigations have been carried out. Chromosome preparations were made from peripheral blood lymphocytes. Fluorescence *in situ* hybridization (FISH) revealed reciprocal translocations and/or inversions that unequivocally distinguish the karyotypes of *Trachypithecus* and *Pygathrix*. DNA sequences were generated from a region of the maternal inherited mitochondrial genome and two autosomal loci.

The hybrid status of the female offspring could be most clearly defined as *Pygathrix nemeaus* (pat) x *Trachypithecus laotum hatinhensis* (mat) with a 44,XX karyotype.

Nghiên cứu về nhiếm sắc thể và phân tử của con lai giữa loài vọoc chà vá chân nâu (*Pygathrix nemaeus*) và loài vọoc Hà Tĩnh (*Trachypithecus laotum hatinhensis*)

Tóm tắt

Bốn cá thể đực của loài vọoc chà vá chân nâu (*Pygathrix nemaeus*) và một đàn vọoc Hà Tĩnh (*Trachypithecus laotum hatinhensis*) được thả chung trong khu bán hoang dã rộng 5 hecta tại Trung tâm Cứu hộ Linh trưởng Nguy cấp, Vườn Quốc gia Cúc Phương. Một tháng sau khi thả, các cá thể

chà vá tiếp cận đàn vọoc Hà Tĩnh, giao phối giữa cá thể đực chà vá và cá thể cái vọoc Hà Tĩnh đã được quan sát. Quá trình mang thai từ 205-207 ngày, một cá thể con lai đã được sinh.

Màu sắc lông của con lai ban đầu giống với màu sắc lông của loài chà vá nhiều hơn, tuy nhiên sau 4 tháng màu lông chuyển toàn bộ sang sắc đen. Một số đặc điểm giống với loài chà vá vẫn hiện hữu như: hình dáng kiểu lông trên đầu, đuôi dài và có màu kem xám, có túm lông đuôi.

Nghiên cứu về hệ thống nhiễm sắc thể được phân tích dựa trên tế bào máu Lympho ngoại biên. Chiếu hình quang cho thấy có hiện tượng trao đổi đoạn và/hoặc nghịch đoạn đã xảy ra một cách rõ rệt trên những nhiễm sắc thể nhuộm thuộc *Trachypithecus* và *Pygathrix*. Trình tự ADN (DNA) đã được tổng hợp tại một vùng của gien ti thể di truyền theo dòng mẹ và hai nhiễm sắc thể thường.

Tình trạng lai của cá thể cái được diễn đạt như sau: *Pygathrix nemaeus* (cha) x *Trachypithecus laotum hatinhensis* (mẹ) với 44 NST, XX.

Introduction

On March 19, 2002 four male red-shanked douc langurs (*Pygathrix nemaeus*) were released to a five hectare electric fenced semi-wild enclosure at the Endangered Primate Rescue Center, Vietnam. The semi-wild enclosure comprises a limestone hill with typical primary limestone forest and vegetation.

The douc langurs had been confiscated from the illegal animal trade and the age of the animals was estimated to between five and six years based on comparisons to a number of red-shanked douc langurs born and raised at the EPRC.

In January 2003 a group of Hatinh langurs (1,3) (*Trachypithecus laotum hatinhensis*) was released to the same area. All these animals were also confiscated and the ages estimated to (male: 8 years, females: 3, 7 and 10 years).

The Hatinh langur group and the douc langur "bachelor-group" formed separate stable social units, similar to groups in the wild. Each species moved and foraged as a single group in the enclosure. After one month, the douc langurs approached the Hatinh langur group, and the male Hatinh langur began keeping his distance from the group. Grooming between douc langur males and Hatinh langur females was occasionally observed and increased. On March 22, 2003 copulation of a douc langur male and a Hatinh langur female was observed. Two days later all four douc langurs were isolated in a cage inside the semi-wild area and translocated back to a cage at the Endangered Primate Rescue Center, outside the semi-wild area.

A hybrid from the species - a female – was born on October 14, 2003. The gestation period for the hybrid was at least 205-207 days. Urine analyses from douc langurs indicate the species' gestation period is 210 days (Lippold, 1981), though a shorter length of 165-190 days has been suggested (Benirschke, http:medicine.uscd.edu/cpa). The gestation period of Hatinh langurs is estimated to about 180 days (Nadler, pers. obs.) and is most probably similar to the closely related Francois' langur (*Trachypithecus francoisi*) which is 184+15 days (Mei Qui Nian & Lai Mao Qing, 1998).

Description of the *Pygathrix nemaeus* x *Trachypithecus laotum hatinhensis* hybrid

The coloration of the newborn hybrid more closely resembled a douc langur, but changed over a period of about four months to completely black (Fig. 1). Newborn Hatinh langurs have a bright golden-yellow fur. Only the tail is dark chocolate-brown. The face of a young Hatinh langur is light

Fig. 1. Hybrid red-shanked douc langur (*Pygathrix nemaeus*) x Hatinh langur (*Trachypithecus laotum hatinhensis*) four months old. Photo: T. Nadler.

Fig. 2. Hatinh langur (*Trachypithecus laotum hatinhensis*) one month old. Photo: T. Nadler.

flesh-colored (Fig. 2). The coloration of newborn douc langurs is more similar to adults: most of the body is grey, with a darker, blackish line on the back along the spine and reddish-grey legs. The face of a young douc langur is variable, mostly slate grey with yellow patches, often with yellow eye rings but can be also totally slate grey (Fig. 3 and 4).

Fig. 3,4. Young douc langurs (Pygathrix nemaeus) with different face coloration. Photo: T. Nadler.

The fur color of the hybrid changed to completely black after four months, a time similar to that of Hatinh langurs. During this time the cheeks changed to light grey, not to white like in Hatinh langurs. The length of the hair on the cheeks was longer but didn't form the typical whiskers of the douc langurs. The fur on the head formed the typical cap of the doucs and not the crest of the Hatinh langurs (Fig. 1, 5). Nostril form is also intermediate between the features of the parental species (Fig. 6). The tail length of 78 cm is intermediate between douc and Hatinh langurs (*Pygathrix* 55,6 cm; 52-58 cm, n=6 adult females [EPRC]; *Trachypithecus* 85,3 cm; 78-90 cm; n=7 adult females [EPRC]), but the tassel on the end more closely resembles that of doucs (Fig. 7). The brachial index and the crural index (fore and hind limbs, respectively) correspond to

Fig. 5. Adult hybrid (four years old). Photo: T. Nadler.

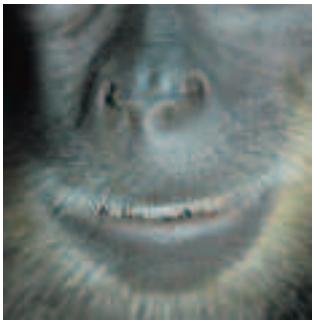

Fig. 6. The form of the nostril is intermediate between the features of the parental species. Photo: T. Nadler.

Fig. 7. Adult hybrid (four years old). Photo: T. Nadler.

Trachypithecus but the proportions between the fore limbs and hind limbs (humerofemoral and intermembral indices, respectively) are intermediate (Table 1).

Table 1. Long bone proportions.

	Pygathrix nemaeus	*Trachypithecus laotum hatinhensis*	Hybrid (female)
Brachial Index (Radius x 100/Humerus)	**106.6** (100-109.5) n=4 ad. females (Nadler, unpubl.) **97-107** (Groves, 1970) **104** (100-107) n=4 (sex?) (Napier & Napier, 1967)	**100.6** (100-103) n=5 ad. females (Nadler, unpubl.)	**100.0**
Crural Index (Tibia x 100/Femur)	**87.0** (82-90.5) n=5 ad. females (Nadler, unpubl.) **88** (Napier & Napier, 1967)	**92.9** (88.6-97.5) n=5 ad. females (Nadler, unpubl.)	**93.8**
Humerofemoral Index (Humerus x 100/Femur)	**88.1** (84-92.9) n=5 ad. females (Nadler, unpubl.)	**79.5** (72.7-85.0) n=5 ad. females (Nadler, unpubl.)	**83.3**
Intermembral Index ([Humerus+Radius] x 100/ [Femur+Tibia])	**97.1** (92.3-101.3) n=5 ad. females (Nadler, unpubl.) **93** (92-94) n=3 (sex ?) (Napier & Napier, 1967) **92-96** (Groves, 1970)	**82.2** (77.1-87.0) n=5 ad. females (Nadler, unpubl.)	**86.0**

Material and Methods

Chromosome preparations

Chromosome preparations were made from peripheral blood lymphocytes according to standard methods, with minor modifications (Schempp & Meer 1983). During the last 7 – 8 hours before harvesting, bromodeoxyuridine (BrdU) was added to the culture. As a result, thymidine was incorporated into early-replicating and BrdU into late-replicating chromosomal segments, allowing the presentation of RBA-banding pattern on metaphase chromosomes (ISCN 2005). Slides carrying interphase cells and metaphase spreads were dehydrated in a series of concentrations of ice-cold ethanol then air-dried and stored at –80°C. Before using for in situ hybridization, the slides were dehydrated again and then air dried.

Fluorescence in situ hybridization (FISH)

Prior to FISH, the slides were treated with RNase followed by pepsin digestion as described (Ried et al. 1992). FISH followed the method described by Schempp et al. (1995). Chromosome in situ suppression (CISS) was applied to human whole-chromosome painting (WCP) libraries (Jauch et al. 1992). After FISH the slides were counterstained with DAPI (0.14 µg/ml) and mounted in Vectashield (Vector Laboratories). Preparations were evaluated using a Zeiss Axiophot epifluorescence microscope equipped with single-bandpass filters for excitation of red, green, and blue (Chroma Technologies, Brattleboro, VT). During exposures, only excitation filters were changed allowing for pixel-shift-free image recording. Images of high magnification and resolution were obtained using a black-and-white CCD camera (Photometrics Kodak KAF 1400; Kodak, Tucson, AZ) connected to the Axiophot. Camera control. Digital image acquisition involved the use of an Apple Macintosh Quadra 950 computer.

Molecular genetics

To further confirm the hybrid status of the study specimen, DNA sequences were generated from a region of the maternal inherited mitochondrial genome and two autosomal loci. Since the study specimen is a female, Y-chromsomal loci were not studied. DNA was extracted from blood with the Qiagen mini-kit following recommendations of the supplier. Loci were amplified via PCR using standard PCR conditions. For the amplification of the hypervariable region I of the mitochondrial D-loop, the oligonucleotide primers 2068 and 2270 were used. The two autosomal loci (transition protein 2 and transthyretin, intron 1) were amplified with the oligonucleotide primer pairs 5'-GCA GGT GTA CAA AAC CAA G-3'/5'-GTC TCA TTA GTT GGA TTT CC-3' and 5'-GGC CCT ACG GTG AGT GTT-3'/5'-ACT TTG ACC ATC AGA GGA CA-3', respectively. PCR reactions were identical for all amplifications and included a predenaturation step at 94°C for 2 min., followed by 40 cycles consisting of denaturation at 94°C for 1 min., annealing at 58°C for 1 min., and elongation at 72°C for 1 min. At the end, a final elongation step at 72°C for 5 min. was added. PCR products were run on agarose gels and excised from the gel. After purification with the Qiagen gel extraction kit, PCR products were sequenced on an ABI3100 capillary sequencer. To evaluate the hybrid status of the study specimen, the generated sequences were compared with those obtained from pure *Pygathrix nemaeus* and *Trachypithecus laotum hatinhensis* individuals.

Results and Discussion

Asian leaf-eating monkeys (Colobinae) have rather conserved karyotypes. With the exception of the proboscis monkey (*Nasalis larvatus*; 2n = 48), all species of *Trachypithecus* and *Pygathrix* investigated have the diploid chromosome number of 2n = 44 (Bigoni et al. 1997a;1997b; 2003; 2004; Nie et al. 1998; Wienberg 2005). Fluorescence in situ hybridization (FISH) using human whole-chromosome paints (WCP) revealed reciprocal translocations and/or inversions that unequivocally distinguish the karyotypes of *Trachypithecus* and *Pygathrix*.

First, a reciprocal translocation homologous to human 6/16 appears to be a distinguishing characteristic of the genus *Trachypithecus* (Bigoni et al. 1997a; 1997b; Nie et al. 1998), while this translocation is absent in *Pygathrix* (Bigoni et al. 2004). Applying human WCP 6 and WCP 16 we could demonstrate this rearrangement in our female *Trachypithecus laotum hatinhensis* (TLA) individual, mother of our putative hybrid offspring: both chromosome pairs 15 and 18 exhibit the rearranged painting pattern of human 6/16 (Fig. 8a). However, in our putative female hybrid offspring this reciprocal painting pattern appeared only in the haploid state marking TLA chromosome 15 and 18, while one larger submetacentric and one small metacentric were painted in total with WCP 6 and WCP 16, respectively (Fig. 8b). Indeed, the larger submetacentric chromosome painted by WCP 6 is characteristic for chromosome 2, and the small metacentric chromosome painted by WCP 16 is characteristic for chromosome 19 of *Pygathrix nemaeus* (Bigoni et al. 2004).

Furthermore, all Asian colobines studied so far share a reciprocal translocation of homologues of human chromosomes 1 and 19 resulting in chromosomes 4 and 5 of *Trachypithecus* (Nie et al. 1998), and in chromosomes 8 and 10 of *Pygathrix* (Bigoni et al. 2004). In *Pygathrix* the primitive ancestral translocation pattern is found, whereas a derived and more complex alternating painting pattern is seen on chromosome 5 of *Trachypithecus*. This derived complex pattern that can be explained by pericentric inversion should have occurred in *Trachypithecus* after the divergence from *Pygathrix* (Bigoni et al. 2003; 2004). Using human WCP 1 and WCP 19 as painting probes the reciprocal translocation pattern on chromosomes 4 and 5 becomes clearly visible in our female *Trachypithecus laotum hatinhensis* (TLA) individual (Fig. 8c). In addition the alternating 1/19 pattern on both chromosomes 5 of TLA (Fig. 8c) confirms the pericentric inversion specific for *Trachypithecus*. In our putative hybrid offspring the derived alternating 1/19 painting pattern specific for *Trachypithecus* appears only on one chromosome, TLA 5 (Fig. 8d), while the primitive ancestral 1/19 painting pattern specific for *Pygathrix* is seen on the other chromosome, PNE 10 (Fig. 8d).

The molecular genetic study provides further evidence for the hybrid status of the study specimen and confirms the findings obtained from the chromosomal investigations. Since the mitochondrial genome is solely maternally inherited, the hybrid should carry only the mitochondrial genome of the mother. In fact, the sequence of the hypervariable region I of the D-loop of the putative hybrid is closely related or even identical with those generated from several *Pygathrix nemaeus* individuals, but differs greatly from *Trachypithecus laotum hatinhensis* haplotypes. In contrast to the mitochondrial genome, autosomal loci are inherited by both parents, so that in F1 hybrids alleles of both parents should be traceable. For this hybrid, we obtained sequences from two autosomal loci, which in fact carry alleles of both *Pygathrix nemaeus* and *Trachypithecus laotum hatinhensis*, so that a hybrid status of the study specimen is confirmed.

In conclusion, the cytogenetic and chromosomal painting as well as the molecular genetic studies shed light on the hybrid status of this female offspring in question. Although no material from the paternal *Pygathrix nemaeus* individual was available for our cytogenetic studies, the hybrid

status of the female offspring could be most clearly defined as *Pygathrix nemeaus* (pat) x *Trachypithecus laotum hatinhensis* (mat) with a 44,XX karyotype.

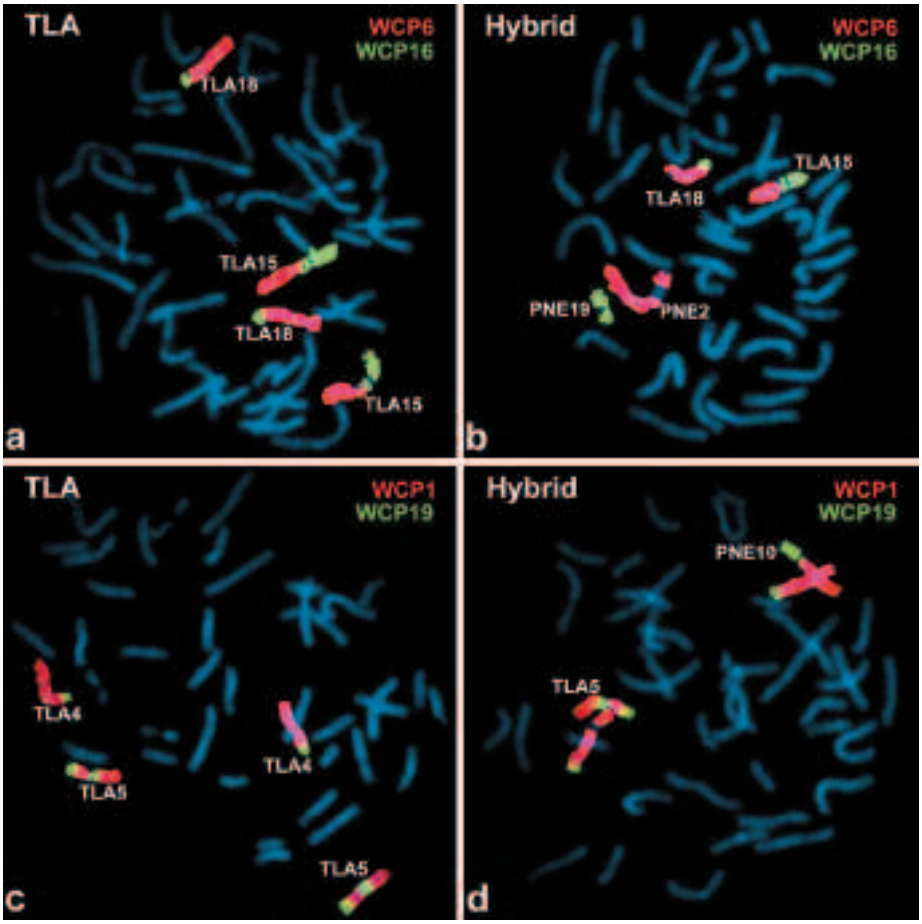

Fig. 8. FISH with human painting probes (WCP) to metaphase spreads of female *Trachypithecus laotum hatinhensis* (TLA) and the female hybrid offspring (Hybrid).

a. Rearranged pattern of human 6/16 on both chromosome pairs 15 and 18 of TLA.

b. The hybrid offspring shows rearranged 6/16 pattern only on one TLA chromosome 16 and 18, respectively, derived from the mother. The larger submetacentric chromosome painted in toto by WCP 6 characterizes chromosome 2 of *Pygathrix nemaeus* (PNE2), and the small metacentric one painted by WCP 16 characterizes chromosome 19 of *Pygathrix nemaeus* (PNE 19), derived from the father of the hybrid.

c. Both chromosomes TLA5 indicate the derived alternating 1/19 painting pattern specific for *Trachypithecus*.

d. The hybrid offspring shows only one chromosome TLA5, derived from the mother. PNE10 shows the primitive ancestral 1/19 painting pattern specific for the paternal *Pygathrix nemaeus*.

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