



Chromosome Painting Shows That *Pygathrix nemaeus* Has the Most Basal Karyotype among Asian Colobinae

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*We mapped the chromosomal homology of *Pygathrix nemaeus* (douc) with human and other primates by in situ hybridization of human chromosome paints. The synteny of 3 human chromosomes (1, 2, 19) is fragmented in the douc karyotype and the 23 human probes (autosomes plus X) provided 26 signals. There are associations between human chromosomes 14/15, 21/22, and 1/19. Human chromosomes 1 and 19 are divided in two segments and associated on douc chromosomes 8 and 10. The fragmentation and association of human chromosomes 1 and 19 is best explained as the result of a reciprocal translocation, which occurs in all documented Asian colobines studied, but not in the African species *Colobus guereza*. However, the homologs to douc chromosome 10 in all other Asian documented colobines show an additional pericentric inversion. Our results indicate that *Pygathrix nemaeus* is karyologically the most conservative colobine species yet studied and that this species probably diverged early after the separation of Asian and African Colobinae. The data reinforce the monophyly of the Colobinae and their division into an African and an Asian clade.*

KEY WORDS: primates; *in situ* hybridization; comparative mapping; phylogeny; evolution.

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INTRODUCTION

Recent research links chromosomal rearrangements to molecular divergence and stresses the important role of chromosomal events in the speciation process (Navarro and Barton, 2003; Rieseberg and Livingstone, 2003). Therefore, it is likely that karyological differences between species are significant and can shed light on the evolutionary history and phylogeny of primates. Fluorescence *in situ* hybridization (FISH) of DNA probes specific to whole chromosomes, chromosome painting, is the method of choice to establish chromosomal homology between species. Precise knowledge of chromosomal homology is essential if cytogenetics is to contribute to phylogenetics and taxonomy. Mapping chromosomal homology between species is also an important first step to understand the mechanisms of chromosomal evolution and the role of chromosomal rearrangements in speciation.

Molecular cytogenetics is an especially valuable tool in the phylogenetic reconstruction of species whose natural history is as poorly understood as that of colobine monkeys. There is no consensus about the evolutionary and taxonomic relationships within the group. Traditional studies were based mostly on morphological traits that may not be highly diagnostic. For example, one widely used character is pelage color, which in colobines is highly variable, even during ontogeny, and therefore of unpredictable taxonomic value (Jablonski, 1995).

One topic of debate involves the genus *Pygathrix*, which includes a group of species with discontinuous distribution at the periphery of the Asian colobine-range (Brandon-Jones, 1996). Groves (1993) placed 5 species in *Pygathrix*: *Pygathrix nemaeus* (douc) and the 4 species of the subgenus *Rhinopithecus* (snub-nosed langur), *R. avunculus*, *R. bieti*, *R. brelichi* and *R. roxelana*. Oates, Davies, and Delson (Delson, 1994) also described *Rhinopithecus* as subgenera of *Pygathrix*. However, Jablonski (1995) and Yan-Zhang *et al.* (1993) separated *Pygathrix* and *Rhinopithecus* into two genera, a view supported also by some molecular data (Wang *et al.*, 1997) and accepted in the revised classification of Groves (2001). Other molecular studies described *Pygathrix* and *Rhinopithecus* as only distantly related sister taxa (Collura *et al.*, 1996) or indicated that they are not sister-taxa at all (Zhang and Ryder, 1998).

Whichever way the genus *Pygathrix* is defined, the affiliation with the other colobine genera is unclear. Despite the fact that little is known about the phylogenetic relationships of the Asian colobines (Morales *et al.*, 1999), *Pygathrix* and *Nasalis* are commonly included in the odd-nosed group (Groves, 2001) and often considered to be the most conservative genera (Bennett and Davies, 1999; Strasser and Delson, 1987). Groves (1989) proposed the separation of *Nasalis* as sister taxa to all the other Asian Colobinae,

but noticed that *Pygathrix* and *Nasalis* share an intermembral index different from all the other Asian species. Recent molecular data on mtDNA presented a possible sister relationship between *Pygathrix* and *Nasalis* (Morales *et al.*, 1999). Classical molecular studies of Sarich (1970) on four genera of Colobinae (*Colobus*, *Pygathrix*, *Nasalis* and *Presbytis*), offered a different picture, which seemed to indicate that *Nasalis* and *Presbytis* share a common ancestor after divergence from *Colobus* and *Pygathrix*.

Recent chromosomal data obtained by fluorescent *in situ* hybridization techniques (FISH) have improved our knowledge of phylogenetic relationships in primate species (Neusser *et al.*, 2001; Stanyon *et al.*, 2000, 2001). In particular, cytogenetic data contributed valuable clues regarding the evolution of the Colobinae. Painting human chromosomal probes on metaphases of *Colobus guereza* (Bigoni *et al.*, 1997b), *Trachypithecus cristatus* (Bigoni *et al.*, 1997a), *T. francoisi*, *T. phayrei* (Nie *et al.*, 1998) and *Nasalis larvatus* (Bigoni *et al.*, 2003) supported Colobinae monophyly and their division into an African and an Asian clade. *Nasalis larvatus*, often previously considered to be primitive for a high chromosomal number ($2n = 48$) and isolated from the other Colobinae (Groves, 1989), appears clearly derived karyologically, and phylogenetically nested within the Asian Colobinae (Bigoni *et al.*, 2003).

We established the chromosomal homology of the douc with human and other primates by *in situ* hybridization of human chromosome paints to douc metaphase chromosomes. Our results indicate that *Pygathrix namaeus* is karyologically the most conservative colobine species studied and that it probably diverged early after the separation of Asian and African Colobinae. Our data reinforce the monophyly of the Colobinae and their division into an African and an Asian clade.

METHODS

Metaphase chromosomes from fibroblast cells of a captive born male *Pygathrix namaeus* (ZSSD # KB6946). Culture techniques and chromosome preparation followed standard procedures. To facilitate chromosome identification we G-banded most chromosome preparations before *in situ* hybridization (Klever *et al.*, 1991), and DAPI-banding concurrently with *in situ* hybridization also facilitated chromosome identification. The karyotype numbering and arrangement are per Bogart and Kumamoto (1978).

Hybridizations

Human chromosome specific probes were made by degenerate oligonucleotide primed PCR (DOP-PCR) from flow sorted chromosomes using PCR primers, amplification and labeling conditions as described by Telenius

et al. (1992), Stanyon *et al.* (1999) and Bigoni *et al.* (2003). We used the same primers (6MW) (Telenius *et al.*, 1992) in the primary reaction and to label the chromosome paints with Cy5-dUTP (Amersham), Rodamine 110-dUTP and Texas-Red-dUTP (both from Molecular Probes) or biotin-dUTP (Roche).

We performed hybridization of human DNA probes (chromosome paints), on douc chromosomes as reported by Bigoni *et al.* (2003). Biotin labeled probes were detected with avidin conjugated to FITC. We recorded images directly on photographic film or via computer image processing.

We followed the nomenclature of Groves (1993) for generic and specific nomina, regardless of their designations in original publications.

RESULTS

The karyotype of *Pygathrix nemaeus* has a diploid number of $2n = 44$ (Fig. 1). All autosomes except one pair are metacentric or submetacentric. One pair of submetacentric chromosomes ($n. 21$) bears the NOR (nucleolar organizer region). The X chromosome is typical for most mammalian species and the Y is a small acrocentric.

The hybridization of all human DNA paints (autosomes and X) provided bright signals on the chromosomes of the douc (Fig. 2). The human probes were divided in 26 signals (Fig. 1). Sixteen human chromosomes (3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, 18, 20, X) painted completely one single douc chromosome. Human chromosomes 14 and 15 were associated on douc chromosome 4. Human chromosomes 21 and 22 were associated on douc chromosome 21: the marker chromosome pair where the NOR is localized.

The synteny of three human chromosomes (1, 2, 19) is fragmented in the douc karyotype. Human probe 2 painted douc chromosomes 12 and 13. Human probes 1 and 19 are both divided in two segments and associated on douc chromosomes 8 and 10.

DISCUSSION

We confirmed that the diploid karyotype number of *Pygathrix nemaeus* is $2n = 44$ (Bogart and Kumamoto, 1978; Hsu and Benirschke, 1975b; Wurster and Benirschke, 1969). The diploid number $2n = 44$ is common to all the other species of the Colobinae (Bigoni, 1995; Bigoni *et al.*, 1997a,b; Chiarelli, 1963; Dutrillaux *et al.*, 1981, 1984; Hsu and Benirschke, 1970, 1971, 1973), with the exception of *Nasalis larvatus* ($2n = 48$) (Bigoni *et al.*, 2003; Chiarelli, 1966; Hsu and Benirschke, 1975a).

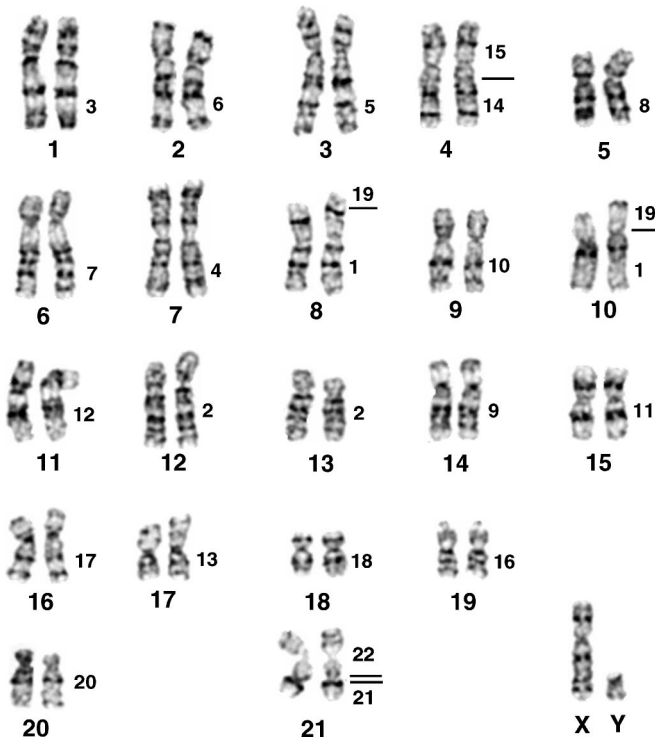


Fig. 1. G-banded karyotype of *Pygathrix nemaeus*. Chromosomes are numbered below and the homology to human chromosome is indicated to the right. The karyotype numbering and arrangement are per Bogart and Kumamoto (1978). Chromosome 21 is the NOR-bearing marker chromosome.

The *in situ* hybridization data allowed us to establish the homologies between human chromosomes and the *Pygathrix nemaeus* karyotype. The comparisons of the karyotype of *Pygathrix nemaeus* with the karyotype of other Colobinae and Primates show that the douc has a highly conserved primate karyotype and the most conservative genomic structure yet described in the colobine lineage.

Associations of Human Chromosomes in *Pygathrix nemaeus*

Two douc chromosomes show associations of human chromosomes. The association of human chromosomes 14 and 15 is on douc chromosome 4. The same association occurs in all Catarrhini except the Hominoidea and is considered ancestral for placental mammals (Murphy *et al.*, 2001).

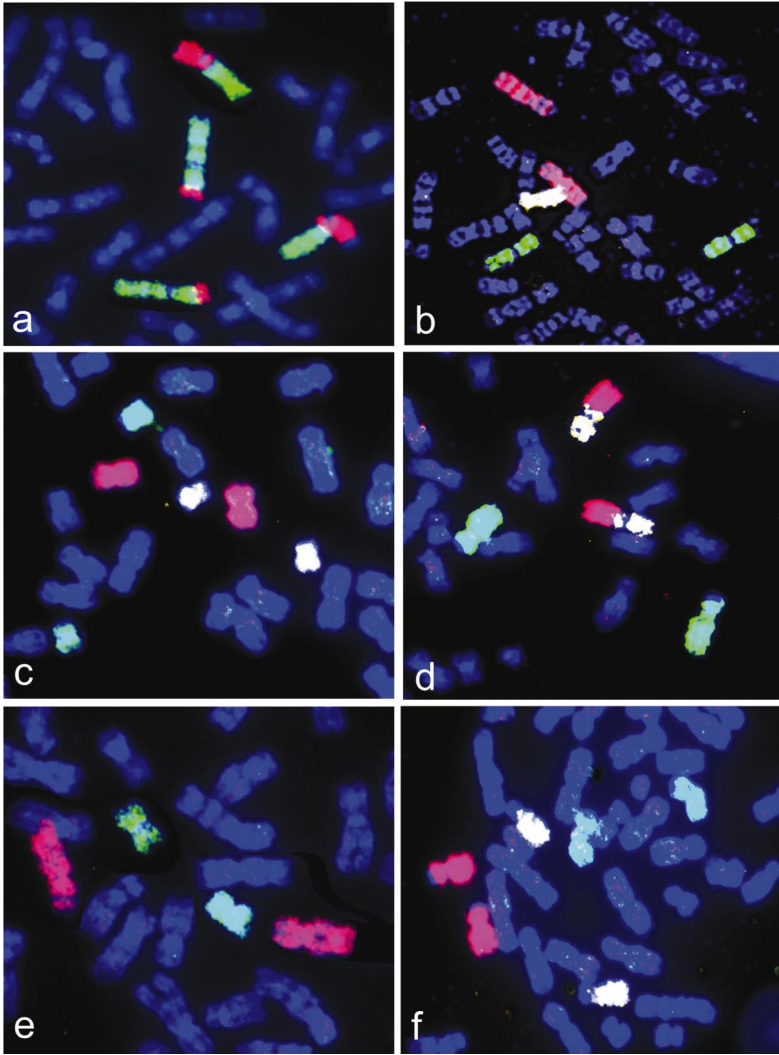


Fig. 2. Hybridization of human paints on *Pygathrix nemaeus* chromosomes a) 1 = green, 19 = red b) 4 = green, 5 = red, c) 6 = green, 11 = red, 18 = yellow, d) 7 = green, 14 = red, 15 = yellow, e) 10 = green, 17 = red, 9 = yellow.

The association of human chromosomes 21 and 22 forming the marker chromosome occurs in all documented Asian colobines (Bigoni, 1995; Bigoni *et al.*, 1997a, 2003; Nie *et al.*, 1998), and also in the African species *Colobus guereza* (Bigoni *et al.*, 1997b). It appears to be one of the chromosomal

rearrangements characterizing the Colobinae, while in the Cercopithecinae the association of human chromosomes 20 and 22 is common (Stanyon *et al.*, 1995).

Human Syntenic Groups Fragmented in *Pygathrix nemaeus*

When a human paint is divided in two or more segments, the genetic synteny is not maintained. The FISH data show that more human syntenic groups are fragmented, as human paintings of chromosomes 1, 2 and 19 are each present on two different douc chromosomes. Human chromosome paint 2 is divided on two douc chromosomes (12 and 13) as expected, since it is well-known that an apomorphic tandem fusion gave origin to human chromosome 2. The other human syntenic groups fragmented are homologous to human chromosomes 1 and 19. Both chromosomes are divided in two segments and are associated on douc chromosomes 8 and 10. The fragmentation and association of human chromosomes 1 and 19 can be explained with a reciprocal translocation that produced the douc chromosomes. This association occurs in all documented Asian Colobinae, but not in the African species *Colobus guereza*, in which there are different translocations (Bigoni *et al.*, 1997b). However, the karyotypes of *Trachypithecus cristatus*, *T. francoisi*, *T. phayrei* and *Nasalis larvatus* show a more complicated pattern of four alternating segments of human chromosome paints 1 and 19 on the same colobine chromosome (Bigoni *et al.*, 1997a, 2003; Nie *et al.*, 1998). The most parsimonious explanation is that a reciprocal translocation occurred in the lineage of the Asian colobines and distinguishes them from the African colobines. Other rearrangements such as inversions may provide distinguishing traits between Asian and African colobines. *Pygathrix nemaeus* shows the primitive reciprocal translocation between 1 and 19 that was followed by a pericentric inversion linking the *Trachypithecus* with *Nasalis larvatus*.

The number of segments or hybridization signals is a good indicator of the evolution of the karyotypes with regard to interchromosomal rearrangements. The human paints are split into 26 segments in *Pygathrix nemaeus*, 30 segments in *Nasalis larvatus* and *Trachypithecus cristatus* and 32 segments in *Colobus guereza* (always considering the female karyotype). The same diploid chromosomal number $2n = 44$ shared by *Pygathrix nemaeus*, *Colobus guereza* and *Trachypithecus cristatus* is the result of different interchromosomal rearrangements. Additionally, the karyotypes of *Trachypithecus cristatus*, *T. francoisi* and *T. phayrei* are derived from a reciprocal translocation between homologs to human 6 and 16 (Bigoni *et al.*, 1997a; Nie *et al.*, 1998). The karyotype of *Nasalis larvatus* is also derived from 2 fissions of the homologs to human chromosomes 14 and 6 (Bigoni *et al.*, 2003).

Our results suggest that *Pygathrix nemaeus* is the most conservative of the Asian Colobinae and is phylogenetically basal to all other documented Asian Colobinae.

It would be interesting to collect cytogenetic data regarding *Rhinopithecus* to clarify the phylogenetic position of the snub-nosed langurs and in particular their relationship to *Pygathrix nemaeus*. Use of reciprocal chromosome painting and of subchromosomal probes of decreasing size such as YACs, BACS and cosmids would effectively contribute to the study of colobines and help to define breakpoints, which may have phylogenetic significance.

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