

RESEARCH ARTICLE

Chromosome Painting Shows That the Proboscis Monkey (*Nasalis larvatus*) Has a Derived Karyotype and Is Phylogenetically Nested Within Asian Colobines

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The exceptional diploid number ($2n=48$) of the proboscis monkey (*Nasalis larvatus*) has played a pivotal role in phylogenies that view the proboscis monkey as the most primitive colobine, and a long-isolated genus of the group. In this report we used molecular cytogenetic methods to map the chromosomal homology of the proboscis monkey in order to test these hypotheses. Our results reveal that the *N. larvatus* karyotype is derived and is not primitive in respect to other colobines ($2n=44$) and most other Old World monkeys. The diploid number of $2n=48$ can be best explained by derived fissions of a segment of human chromosomes 14 and 6. The fragmentation and association of human chromosomes 1 and 19 as seen in other Asian colobines, but not in African colobines, is best explained as a derived reciprocal translocation linking all Asian colobines. The alternating hybridization pattern between four segments homologous to human chromosomes 1 and 19 on *N. larvatus* chromosome 6 is the result of the reciprocal translocation followed by a pericentric inversion. *N. larvatus* shares this pericentric inversion with *Trachypithecus*, but not with *Pygathrix*. This inversion apparently links *Nasalis* and *Trachypithecus* after the divergence of *Pygathrix*. The karyological data support the view that Asian colobines, including *N. larvatus*, are monophyletic. They share many linking karyological features separating them from the African colobines. The hybridization pattern also suggests that *Nasalis* is nested within Asian Colobines and shares a period of common descent with other Asian colobines after the divergence of *Pygathrix*. Am. J. Primatol. 60:85–93, 2003. © 2003 Wiley-Liss, Inc.[†]

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INTRODUCTION

Many problems remain concerning the evolution and taxonomy of the colobines, and in particular the phylogenetic position of the proboscis monkey (*Nasalis larvatus*). Even the concept of a geographical division of the colobines into an African clade and an Asian clade [Napier, 1985; Oates et al., 1994], which is supported by both molecular [Collura et al., 1996; Collura & Stewart, 1995; Disotell, 1996; Messier & Stewart, 1997; Page et al., 1999; Sarich, 1970] and morphological studies [Delson, 1992; Strasser & Delson, 1987], has not gone unchallenged [Giusto & Margulis, 1981; Groves, 1989; Peng et al., 1993].

The exceptional diploid number ($2n=48$) of *N. larvatus* [Chiarelli, 1966; Soma et al., 1974; Stanyon et al., 1992] has played a pivotal role in phylogenies that view the proboscis monkey as the most primitive colobine, and a long-isolated genus of the group [Giusto & Margulis, 1981; Groves, 1989; Peng et al., 1993]. Groves [1989] considered *N. larvatus* primitive for a relevant number of morphological characters (for the most part linked to the lack of masticatory specialization seen in other colobines) and for the diploid number. *Nasalis* was placed as a sister species to all other African and Asian colobines, and the Colobidae were divided into two subfamilies: the Nasalinae and Colobinae. Harvati [2000] found support for Groves on the basis of colobine dental eruption sequences. Peng et al. [1993] also claimed that *Nasalis* is the most primitive colobine genus, based on morphological measurements and, again, the chromosome number. It should be noted that in a later work Groves grouped the proboscis monkey with Asian Colobines [Groves, 2001].

On the other hand, molecular studies have provided evidence of a monophyletic Asian clade that includes four lineages: the *Nasalis*, *Rhinopithecus*/*Pygathrix*, *Semnopithecus* (*entellus* and *vetulus*), and *Trachypithecus* (*francoisi*, *obscurus*, and *cristatus*) [Collura et al., 1996]. Zhang and Ryder [1998] supported the idea of a monophyletic Asian clade, and suggested a possible lineage including *Nasalis*, *Rhinopithecus*, and *Pygathrix*.

A number of publications have reported on chromosome painting in colobines [Bigoni, 1995; Bigoni et al., 1997a, b; Nie et al., 1998]. In situ hybridization data suggest that the colobines divided into African and Asian clades. Although both African and Asian colobines have the same diploid number ($2n = 44$), the syntenic associations present in each group differ. Finally, comparisons of the G-banded chromosomes of *N. larvatus* with other primates suggested that the proboscis monkey karyotype is derived and is not primitive [Bigoni, 1995; Stanyon et al., 1992].

In this study we used molecular cytogenetic methods to map the chromosomal homology of the proboscis monkey in order to test these hypotheses. Our results support the view that the *N. larvatus* karyotype is derived and is not primitive in respect to other colobines and most other Old World monkeys. This view is based on both the chromosome number and the syntenies present in the karyotype. We show that, regardless of the derived apomorphic characters, *Nasalis* is closely related to and nested within other species of Asian colobines. We also discuss the position of *vetulus* and suggest that this species should be removed from the genus *Trachypithecus*.

METHODS

Heparinized blood samples from Bagus, a male proboscis monkey (*N. larvatus*), were kindly provided by Wolfram Rietschel, from the Wilhelma Zoo in Stuttgart, Germany. Bagus was born on 15 August 1978 at the Cologne Zoo,

Germany, transferred to the Wilhelma Zoo on 20 July 1990, and died on 7 January 1993. Bagus's mother, a wild-born proboscis monkey from Borneo (the precise location of capture is unknown), was brought to the Cologne Zoo when she was about 3 years old.

Chromosome spreads from PHA-stimulated peripheral blood lymphocytes were prepared according to standard methods [Schempp et al., 1995]. To facilitate chromosome identification, most chromosome preparations were G-banded prior to in situ hybridization [Klever et al., 1991]. DAPI-banding concurrently with in situ hybridization also facilitated chromosome identification. 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 previously described [Stanyon et al., 1999; Telenius et al., 1992]. Chromosomes were sorted with a dual laser cell sorter (FACS Vantage SE; Becton Dickinson). This system allowed a bivariate analysis of the chromosomes by size and base pair composition. About 200 chromosomes were sorted from each peak in the flow karyotypes. Chromosomes were sorted directly into PCR tubes containing 30 μ l of distilled water. The same primers (6MW) [Telenius et al., 1992] were used in the primary reaction and to label the chromosome paints with Cy5-dUTP (Amersham, Piscataway, NJ), Rodamine 110-dUTP, and Texas-Red-dUTP (all from Molecular Probes, Eugene, OR).

About 350 ng of each probe were precipitated along with 10 μ g of human Cot-1 DNA and 10 μ g of salmon sperm DNA (both Invitrogen/Life Technologies, Carlsbad, CA). The DNA was then dissolved in 14 μ l of hybridization buffer, denatured at 80°C for 8 min, and pre-annealed for 90 min. Chromosomes were denatured in 70% formamide, 2XSSC at 65°C for 1 min 30 sec. After hybridizing for 48 hr, the slides were washed at 42°C in 50% formamide in 2XSSC, and then three times in 1XSSC.

We followed the nomenclature of Groves [1993] for genus and species names, regardless of the designations in the original publications, with the exception of *Trachypithecus vetulus*, which we consider as *Semnopithecus vetulus*.

RESULTS

We confirmed that the diploid number ($2n = 48$) of *N. larvatus* is unique among colobines [Chiarelli, 1966; Soma et al., 1974; Stanyon et al., 1992]. All the chromosome are submetacentric or metacentric. One pair of metacentric chromosomes (15) bears the nucleolar organizer region (NOR). The X chromosome is typical for most mammals, while the Y-chromosome is a relatively large submetacentric.

The hybridizations of all human DNA paints, except the Y, provided bright signals on proboscis monkey chromosomes (Fig. 1). The human probes were divided into 30 signals in the proboscis monkey karyotype (Fig. 2). Fourteen human paints (3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 16, 17, 18, 20, and X) each hybridized a single *N. larvatus* chromosome completely.

As expected, the DNA probes specific for human chromosome 2 hybridized two *N. larvatus* chromosomes (8 and 13). It is well known that the human chromosome 2 originated by an apomorphic tandem fusion after the divergence of the human lineage from the African apes [IJdo et al., 1991; Murphy et al., 2001; O'Brien & Stanyon, 1999; Stanyon et al., in press; Wienberg & Stanyon, 1998]. However, the synteny of four other human chromosomes (1, 6, 14, and 19) found fragmented in the proboscis monkey karyotype represents derived conditions in

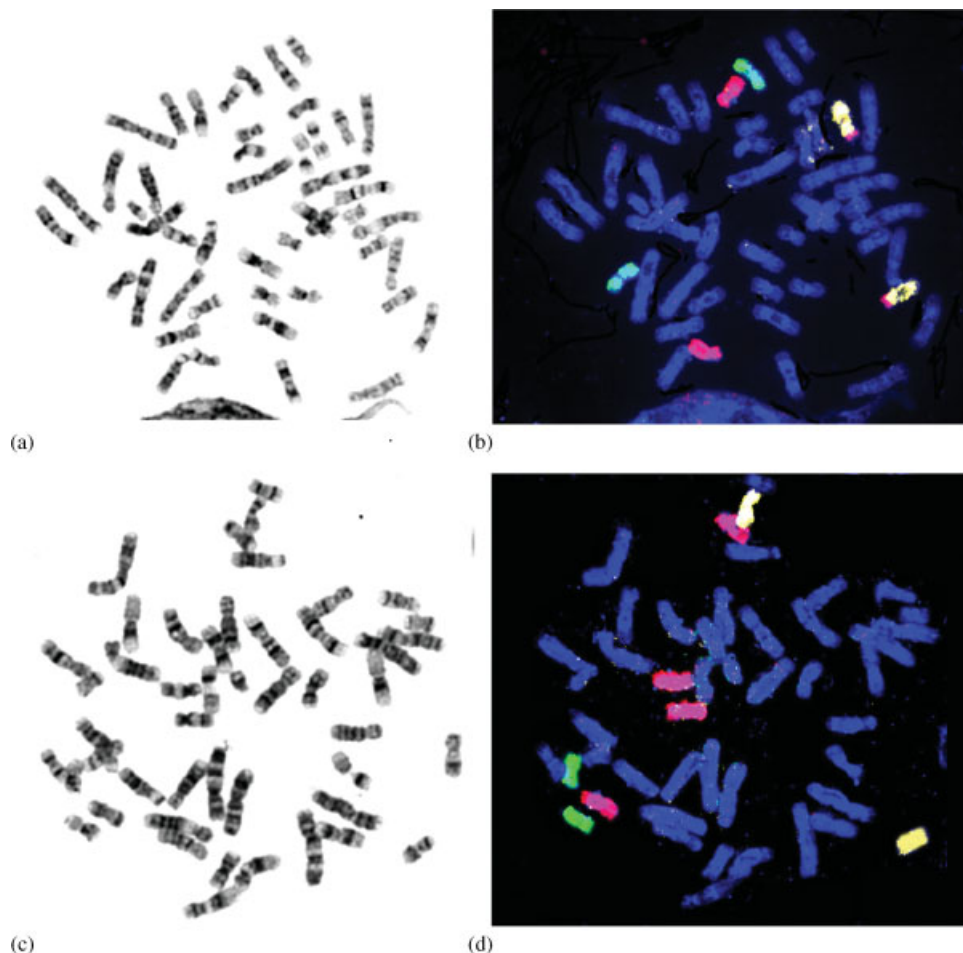


Fig. 1. Examples of G-banding followed by triple in situ hybridizations of human chromosome probes on proboscis monkey (*Nasalis larvatus*) metaphases. **a:** G-banded metaphase. **b:** Human chromosome probes 13 in green (Rodamine 110), 14 in red (Texas Red), and 15 in yellow (Cy5), painting proboscis monkey chromosomes. Note the association of 14 and 15 on proboscis chromosome 17. The other part of human 14 paints the whole proboscis 21. **c:** G-banded metaphase. **d:** Paintings of human chromosome probes 16 in green, 6 in red, and 18 in yellow. Note the fission of the homolog to human chromosome 6 forming proboscis chromosomes 18 and 19.

respect to the ancestral genome of all catarrhine primates [Murphy et al., 2001; O'Brien & Stanyon, 1999; Stanyon et al., in press; Wienberg & Stanyon, 1998].

Human chromosome 6 probes hybridized to proboscis monkey chromosomes 18 and 19. In *N. larvatus* the homolog to human chromosome 14 is fissioned into two pieces: one segment paints proboscis monkey chromosome 21, and a second small segment is found on proboscis chromosome 17 in association with the homolog to human chromosome 15. In many Old World primates and other mammals from diverse orders, human chromosomes 14 and 15 are found syntenically associated on one chromosome. This condition is considered ancestral not only for primates, but also for placental mammals [Murphy et al., 2001].

We confirmed the identification of *N. larvatus* chromosome 5 as a homolog to human 1 [Stanyon et al., 1992; Wimmer et al., 2002]. In addition, we found that

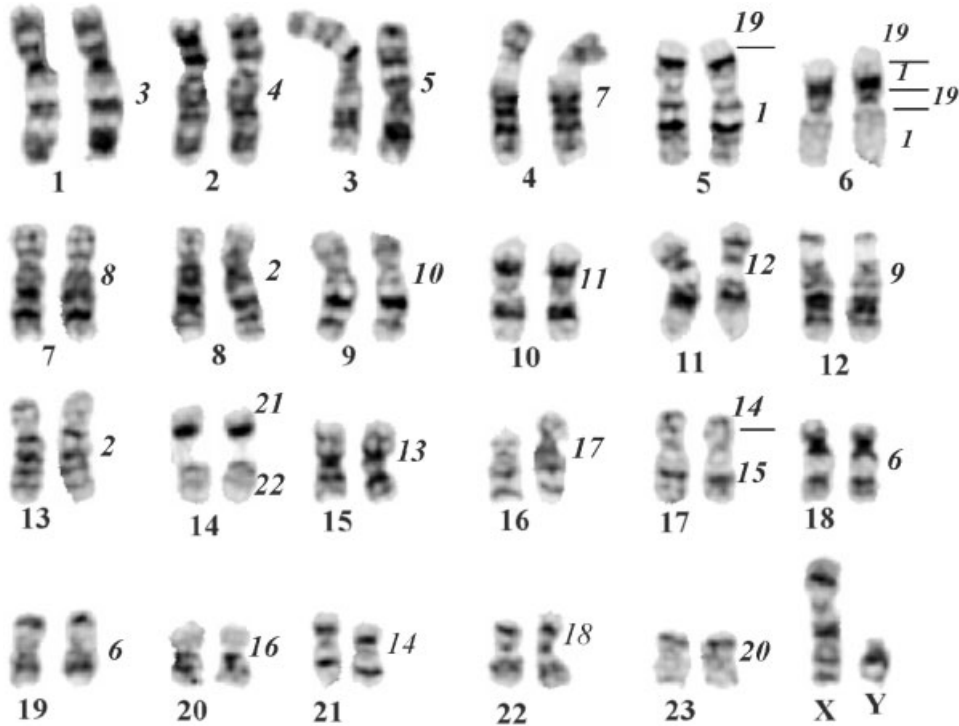


Fig. 2. G-banded karyotype of *Nasalis larvatus* showing the in situ hybridization results. The proboscis monkey chromosomes are numbered below, and the homology with human chromosomes is on the right.

segments homologous to chromosomes 1 and 19 were associated on two *N. larvatus* chromosomes. Because of the alternating signals of human 1 and 19, *N. larvatus* chromosome 6 was divided into four segments.

Human paints 21 and 22 were found associated on *N. larvatus* chromosome 14 (marked chromosome). This association apparently characterizes both African and Asian colobines: together they form the colobine "marked" chromosome. This character distinguishes the Colobinae clade from the Cercopitheciinae, where instead the association of human chromosomes 20 and 22 most often forms the "marked" chromosomes [Stanyon et al., 1995].

DISCUSSION

Comparisons with molecular cytogenetic data in other primates show that the *N. larvatus* genome is derived and is not primitive. Reconstructions of the ancestral catarrhine karyotype indicate that the diploid number was most likely $2n = 46$. The ancestral karyotype would include homologs to human chromosomes 1, 2a, 2b, 3–13, 14/15, 16–22, X, and Y [Stanyon et al., in press]. Four chromosomes found fragmented in the proboscis karyotype (homologs to human chromosomes 1, 6, 14, and 19) are derived with respect to the ancestral catarrhine karyotype. Further, the proboscis diploid number of $2n = 48$ vs. $2n = 44$ in all other colobines can best be explained by derived fissions of human chromosomes 14 and 6. Consequently, the higher diploid number found in *N. larvatus* is not, as mistakenly assumed, a primitive character.

Phylogenetic Implication of the Fission of Human Chromosome 6

Human chromosome 6 is fragmented in *N. larvatus* and found on chromosomes 18 and 19. The human chromosome 6 probe painted only one chromosome in the African colobine species *Colobus guereza* [Bigoni et al., 1997b], and in *Pygatrix nemaeus* [Bigoni, 1995]. G-banding analyses demonstrated that human chromosome 6 is also maintained in some other species of Asian colobines, including *Semnopithecus entellus*, *Presbytis comata*, and *S. vetulus* [Bigoni, 1995]. In *Trachypithecus cristatus* [Bigoni et al., 1997a], *T. francoisi*, and *T. phayrei* [Nie et al., 1998], the segments homologous to human chromosome 6 are involved in an apparent reciprocal translocation with the homolog to chromosome 16, forming two chromosomes with association 6/16. In recent taxonomies, *vetulus* was included in *Trachypithecus* [Groves, 1993; Oates et al., 1994]. On the basis of the reciprocal translocation that links *T. cristatus/francoisi/phayrei*, we suggest that this species could be excluded from the genus *Trachypithecus* and included in *Semnopithecus*. This conclusion is supported by mtDNA data [Collura & Stewart, 1995] (Stewart, personal communication).

We cannot exclude the possibility that the fission of homologs to human chromosome 6 links *N. larvatus* with some *Trachypithecus* species after the divergence of *Presbytis* and *Semnopithecus*. Then *N. larvatus* would show an intermediate stage between all of the colobine species with intact human syntenic group 6 and the genus *Trachypithecus* (excluding *vetulus*) that have two syntenic associations for human 6/16. According to this hypothesis, chromosome 6 would have been fissioned in a common ancestor of *Nasalis* and *Trachypithecus*. After the divergence of *N. larvatus*, two fusion events involving chromosome 6 and 16 homologs would have occurred in the phylogenetic line leading to *Trachypithecus*. This hypothesis is less parsimonious than the alternative hypothesis, which we favor here, that the fissions of chromosome 6 in these taxa are independent events. However, to distinguish between these hypotheses we need to know if the breakpoints in *Nasalis* and *Trachypithecus* are the same, and if the resulting segments are therefore truly homologous. To test these different hypotheses, it is necessary to perform more detailed studies, using such methods as reciprocal chromosome painting, hybridization with subregional probes, and eventually cloning and sequencing of the breakpoints.

Association of Human Chromosomes 14 and 15 and Fission of 14

Human chromosome probes 14 and 15 were found associated on one apparently identical chromosome for all colobine species previously studied [Bigoni et al., 1997a, b; Nie et al., 1998]. In the proboscis monkey, this synteny is fissioned and the homolog to human 14 is found on two different proboscis chromosomes. This apomorphic trait differentiates *N. larvatus* from all other colobines.

Reciprocal Translocation and Inversion of Human Chromosomes 1 and 19

The fragmentation and association of human chromosomes 1 and 19 may be explained as a reciprocal translocation that produced proboscis chromosomes 5 and 6. This apparent reciprocal translocation is found in all Asian colobines studied to date, but it is absent in the African species *Colobus guereza*.

The hybridization pattern on *N. larvatus* chromosome 6 is complex because we identified four alternating segments homologous to human chromosomes 1

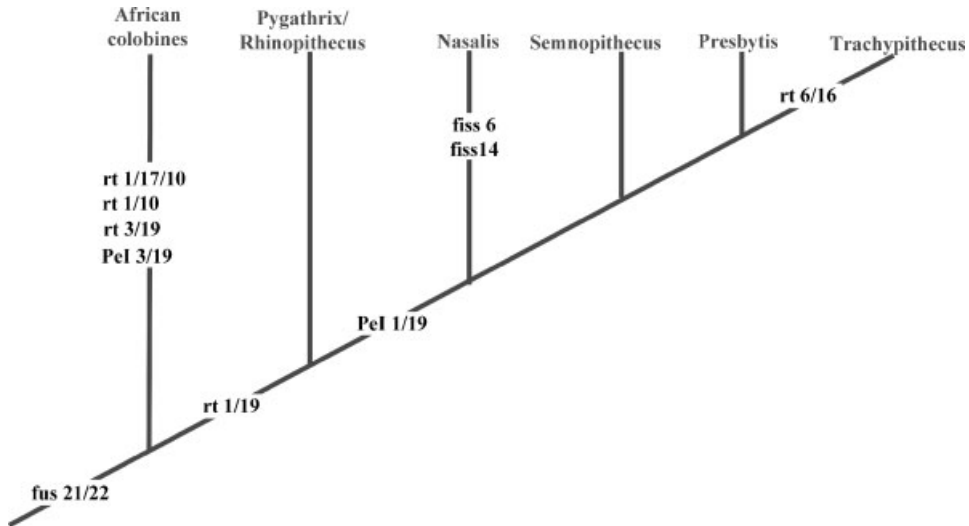


Fig. 3. Phylogenetic tree of the Colobinae based on in situ hybridization data, showing the main chromosomal rearrangements that mark the evolution of their karyotype in different genera. (See text for citations of original articles.) Pel = pericentric inversion; rt = reciprocal translocation; fus = fusion; fiss = fission.

and 19. This pattern is best explained as a reciprocal translocation followed by a pericentric inversion.

An identical alternating pattern was also found in *T. cristatus*, *T. francoisi*, and *T. phayrei* [Bigoni et al., 1997a; Nie et al., 1998]. The karyotype of *Pygathrix nemaeus* shows the reciprocal translocation between 1 and 19, but not the subsequent pericentric inversion [Bigoni, 1995]. *N. larvatus* shares this pericentric inversion with *Trachypithecus*, but not with *Pygathrix*. This chromosomal trait apparently links *Nasalis* and *Trachypithecus* after the divergence of *Pygathrix*.

CONCLUSIONS

Because of its higher diploid number and various morphologic traits, *N. larvatus* has often been considered to be basal to all other colobines [Groves, 1989]. Our data do not support this hypothesis. Instead, the molecular cytogenetic evidence strongly indicates that the proboscis monkey genome is derived. The higher diploid number cannot be considered to be an indicator of primitive characters at the karyological level. The karyological data support the view that Asian colobines, including *Nasalis*, are monophyletic. They share many linking karyological features that separate them from the African colobines. The hybridization pattern also suggests that *Nasalis* is nested within Asian colobines and shares a period of common descent with other Asian colobines after the divergence of *Pygathrix* (Fig. 3).

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