The First Karyological Study and Natural NOR Polymorphism in Banded Langur, *Presbytis femoralis* (Primate, Colobinae)

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**Summary** We report the first polymorphism of nucleolar organizer regions (NORs) and karyological analysis of the banded langur (*Presbytis femoralis*) from Thailand. Blood samples were taken from two male and one female langurs. After standard peripheral blood lymphocytes had been cultured at 27°C for 96 h in the presence of colchicine, the metaphase spreads were performed on a microscopic slide and air-dried. Conventional, GTG-banding and high-resolution techniques were applied to stain the chromosome. The results showed that the diploid chromosome number of *P. femoralis* is 2n = 44, and the fundamental numbers (NF) of both sexes is 88. The types of autosomes are 20 large metacentric, 8 large submetacentric, 2 large acrocentric, 2 medium submetacentic, 4 small metacentric, 4 small submetacentric, and 2 small acrocentric chromosomes. The X chromosome is a large submetacentric chromosome and the Y chromosome is the smallest submetacentric chromosome. In addition, the long arm near the centromere of chromosome pair 17 showed clearly observable NORs. The result showed different sizes of NORs for heteromorphic chromosome pair 17 of all males and female. From the GTG-banding and high-resolution techniques, the number of bands on one set of haploid chromosomes in the *P. femoralis* are 264 and 332, respectively; each chromosome pair could be clearly differentiated. The karyotype formula for *P. femoralis* could be deduced as:

\[ 2n \text{ (diploid)} \ 44 = L_{m}^{20} + L_{m}^{8} + L_{a}^{2} + M_{sm}^{2} + S_{m}^{4} + S_{m}^{4} + S_{a}^{2} \ + \text{sex chromosomes} \]

**Key words** *Presbytis femoralis*, Karyotype, Chromosome, NOR polymorphism.

In the present, the population of wild animals has been decreasing rapidly. Banded langur (*Presbytis femoralis* Raffles, 1821) is one of the species categorized in appendix II by CITES, which is concerned with vulnerable species in the future. The *P. femoralis* belongs to the order primate, family Cercopithecidae and subfamily Colobinae (Lekagul and McNeely 1988, Wilson and Cole 2000, Parr 2003). There are three families, five genera and 13 species found in Thailand. The langur species consists of two genera and four species in Thailand, namely the banded langur (*P. femoralis*), silvered leaf monkey (*Trachypithecus cristatus* Raffles, 1821), dusky leaf monkey (*T. obscurus* Ried, 1837), and Phryre’s leaf monkey (*T. phayrei* Blyth 1847). The common characteristic of the *P. femoralis* is the pelage color, which varies from black to very dark brown to pale gray to pale sepia brown. The under-parts are generally somewhat lighter, and some may have considerable amounts of white on the chest. The inner side of the thigh is conspicuously white.
continuing beyond the knee. The underside of the tail is only slightly paler than the upper parts. There is a white patch on both lips (Lekagul and McNeely 1988, Parr 2003) (Fig. 1).

Langurs belong to a large group of old world monkeys called the colobines (subfamily Colobinae) and are distributed in tropical Asia. The colobines are unique among primates in that they are predominantly leaf eaters and exhibit foregut fermentation. It is generally agreed that the colobines consist of two groups, or clades: the African colobus monkeys and the Asian langur and leaf monkeys (Oates et al. 1994, Morales et al. 1999). Asian colobines have been divided into five or six species groups, where the exact number reported depends on the author (Napier and Napier 1985, Oates et al. 1994, Groves 2001, Brandon-Jones et al. 2004). According to the most recent classifications (Groves 2001, Brandon-Jones et al. 2004), the Asian colobines consist of five species groups. These include langurs (Semnopithecus), leaf monkeys (Trachypithecus), surili (Presbytis), snub-nosed monkeys (Rhinopithecus and Pygathrix), proboscis monkeys (Nasalis), and pig-tailed monkeys (Simias). In south Asia, colobines are represented by two genera: Semnopithecus and Trachypithecus (Karanth 2010).

In the previous studies using classical staining, the diploid number of both African (genus Colobus) and Asian (genus Presbytis) colobines was found to be diploid \((2n) = 44\) (Chiarelli 1963, Ushima et al. 1964). All chromosomes can be divided into metacentric or submetacentric chromosomes according to their centromeric index, except the Asian langurs have a pair of small acrocentric chromosomes that are found to be metacentric chromosomes in the African colobines (Nie et al. 1998). In this study, we provide a detailed description of conventionally stained chromosomes and the distribution pattern of the G-heterochromatin regions. The objective of this study was to investigate and to present for the first time the polymorphisms of the nucleolar organizer regions (NORs) in \(P. \text{femoralis}\) by conventional, GTG-banding and high-resolution techniques. Moreover, the standardizations of chromosome including the chromosome shape and size, karyotype formulating, karyotyping, and idiograming had not been studied for \(P. \text{femoralis}\). In the future, the basic knowledge and cytogenetics of \(P. \text{femoralis}\) could be applied to several studies and especially to their extinction protection.

Materials and methods

Blood samples from the femoral vein were collected from two males and one female, which were kept in Songkhla Zoo, Songkhla Province, Thailand, using aseptic technique. The samples were kept in 10mL vacuum tubes containing heparin to prevent blood clotting, and were cooled on ice until arriving at the laboratory.
**Cell preparation**

The lymphocytes were cultured using the peripheral blood microculture technique adapted from Moorhead *et al.* (1960) and Rooney (2001).

Cell culture: The RPMI 1640 medium was prepared with 2% PHA (Phytohemagglutinin) as a mitogen and kept in blood culture bottles of 5 mL each. A blood sample of 0.5 mL was dropped into a medium bottle and mixed well. The culture bottle was loosely capped, incubated at 37°C under a 5% carbon dioxide environment and regularly shaken in the morning and evening. When reaching harvest time at the 72nd hour of incubation, colchicine was introduced and well mixed, followed by further incubation for 30 min.

Cell harvest: The blood sample mixture was centrifuged at 1,200 rpm for 10 min and the supernatant was discarded. Then, 10 mL of hypotonic solution (0.075 M KCl) was applied to the pellet and the mixture was incubated for 30 min. KCl was discarded with the supernatant after centrifugation again at 1,200 rpm for 10 min. Cells were fixed by a fresh, cold fixative (methanol: glacial acetic acid = 3:1) gradually added up to 8 mL before centrifuging again at 1,200 rpm for 10 min, and the supernatant was discarded. The fixation was repeated until the supernatant was clear and the pellet was mixed with 1 mL of fixative. The mixture was dropped onto a clean and cold slide using a micropipette followed by the air-drying technique. The slide was conventionally stained with 20% stock Giemsa’s solution for 30 min.

**GTG-banding technique**

GTG-banding technique was adapted from Campiranont (2003). The slide was well dried and then soaked in working trypsin (0.025% trypsin EDTA) at 37°C before the termination of trypsin activity by washing the slide with sorensen buffer. The slide was stained with 20% Giemsa’s solution for 30 min.

**High-resolution technique**

High-resolution technique was adapted from Rooney (2001). After the lymphocytes were cultured for 72 h, 0.05 mL of 10⁻⁵ M methotrexate was applied into the cultured lymphocytes to induce synchronization. The mixture was incubated again for 17 h before the methotrexate was discarded with the supernatant by centrifuging at 2,800 rpm. The pellet was mixed with 5 mL of the RPMI 1640 medium and centrifuged at 2,800 rpm. The supernatant was discarded before the cultured cells were released by adding 0.2 mL thymidine and incubating for 5 h and 15 min. The cells were harvested at the exact time and stained by using GTG-banding procedure.

**Chromosomal checks, karyotyping and idiograming**

Chromosome counting was performed on mitotic metaphase cells under a light microscope. Twenty clearly observable and well-spread chromosomes of each male and female were selected and photographed. The length of the short arm chromosome (Ls) and the length of the long arm chromosome (Ll) were measured and used to calculate the length of the total arm chromosome (LT, LT = Ls + Ll). The relative length (RL) and the centromeric index (CI) were estimated. CI was also computed to classify the types of chromosomes according to Chaiyasut (1989). All parameters were used in karyotyping and idiograming.

**Results**

**Conventional staining patterns**

The cytotogenetic study of *P. femoralis* using the standard peripheral blood T-lymphocyte culture demonstrated that the chromosome number is 2n (diploid) = 44, the fundamental numbers (NF, number of chromosome arms) of both sexes is 88 and the number of autosomal arms (NFa) of
both sexes is 84. Autosomes consist of 20 large metacentric, 8 large submetacentric, 2 large acrocentric, 2 medium submetacentric, 4 small metacentric, 4 small submetacentric, and 2 small acrocentric chromosomes. The X chromosome is a large submetacentric chromosome and the Y chromosome is the smallest submetacentric chromosome. The identification of the X chromosome is equivocal, since one or two pairs of autosomes show similar morphology, while the identification of the Y chromosome is very easy (dot chromosome). The *P. femoralis* demonstrated that the chromosome markers are the autosomal pair 1, the largest metacentric chromosome, and the smallest submetacentric Y chromosome. The important karyotype feature of *P. femoralis* is the asymmetrical karyotypes, which were found in all three types of chromosomes (metacentric, submetacentric and acrocentric chromosomes). The largest chromosome is 15 times larger than the smallest chromosome (Figs. 2 and 3).

The chromosome length in centimeters of 20 cells (male and female) in mitotic metaphase was measured. The mean length of the short arm chromosome (Ls) and long arm chromosome (Ll), the mean total length of arm chromosome (LT), relative length (RL), centromeric index (CI), the standard deviation of RL and CI, and the size and type of chromosome are presented in Table 1. The idiogram of *P. femoralis* shows a gradually decreasing length of the chromosomes. The
Fig. 3. Idiogram of banded langur (*Presbytis femoralis*), 2n=44 by conventional staining technique, showing nucleolar organizer region, NOR (arrow).

Table 1. Mean length of short arm chromosome (Ls), mean length of long arm chromosome (Ll), mean length of total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL and CI from metaphase chromosomes in 20 cells of the banded langur (*Presbytis femoralis*), 2n=44.

<table>
<thead>
<tr>
<th>Chromosome pair</th>
<th>Ls</th>
<th>Ll</th>
<th>LT</th>
<th>RL±SD</th>
<th>CI±SD</th>
<th>Chromosome size</th>
<th>Chromosome type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.689</td>
<td>0.929</td>
<td>1.618</td>
<td>0.035±0.003</td>
<td>0.575±0.027</td>
<td>Large</td>
<td>Metacentric</td>
</tr>
<tr>
<td>2</td>
<td>0.321</td>
<td>1.158</td>
<td>1.479</td>
<td>0.032±0.002</td>
<td>0.782±0.019</td>
<td>Large</td>
<td>Acrocentric</td>
</tr>
<tr>
<td>3</td>
<td>0.645</td>
<td>0.834</td>
<td>1.479</td>
<td>0.031±0.002</td>
<td>0.561±0.023</td>
<td>Large</td>
<td>Metacentric</td>
</tr>
<tr>
<td>4</td>
<td>0.500</td>
<td>0.971</td>
<td>1.471</td>
<td>0.031±0.003</td>
<td>0.660±0.083</td>
<td>Large</td>
<td>Submetacentric</td>
</tr>
<tr>
<td>5</td>
<td>0.468</td>
<td>0.738</td>
<td>1.205</td>
<td>0.025±0.002</td>
<td>0.615±0.051</td>
<td>Large</td>
<td>Submetacentric</td>
</tr>
<tr>
<td>6</td>
<td>0.456</td>
<td>0.736</td>
<td>1.193</td>
<td>0.025±0.002</td>
<td>0.625±0.052</td>
<td>Large</td>
<td>Submetacentric</td>
</tr>
<tr>
<td>7</td>
<td>0.411</td>
<td>0.769</td>
<td>1.180</td>
<td>0.025±0.001</td>
<td>0.646±0.046</td>
<td>Large</td>
<td>Submetacentric</td>
</tr>
<tr>
<td>8</td>
<td>0.361</td>
<td>0.760</td>
<td>1.121</td>
<td>0.024±0.001</td>
<td>0.677±0.029</td>
<td>Large</td>
<td>Submetacentric</td>
</tr>
<tr>
<td>9</td>
<td>0.396</td>
<td>0.725</td>
<td>1.111</td>
<td>0.023±0.001</td>
<td>0.655±0.023</td>
<td>Large</td>
<td>Submetacentric</td>
</tr>
<tr>
<td>10</td>
<td>0.500</td>
<td>0.603</td>
<td>1.103</td>
<td>0.023±0.001</td>
<td>0.547±0.023</td>
<td>Large</td>
<td>Metacentric</td>
</tr>
<tr>
<td>11</td>
<td>0.364</td>
<td>0.704</td>
<td>1.068</td>
<td>0.023±0.001</td>
<td>0.658±0.029</td>
<td>Large</td>
<td>Submetacentric</td>
</tr>
<tr>
<td>12</td>
<td>0.360</td>
<td>0.698</td>
<td>1.058</td>
<td>0.022±0.002</td>
<td>0.667±0.047</td>
<td>Large</td>
<td>Submetacentric</td>
</tr>
<tr>
<td>13</td>
<td>0.480</td>
<td>0.536</td>
<td>1.016</td>
<td>0.021±0.001</td>
<td>0.532±0.030</td>
<td>Large</td>
<td>Metacentric</td>
</tr>
<tr>
<td>14</td>
<td>0.405</td>
<td>0.593</td>
<td>0.998</td>
<td>0.021±0.001</td>
<td>0.614±0.030</td>
<td>Large</td>
<td>Submetacentric</td>
</tr>
<tr>
<td>15</td>
<td>0.349</td>
<td>0.584</td>
<td>0.933</td>
<td>0.020±0.002</td>
<td>0.619±0.078</td>
<td>Large</td>
<td>Submetacentric</td>
</tr>
<tr>
<td>16</td>
<td>0.256</td>
<td>0.621</td>
<td>0.878</td>
<td>0.018±0.001</td>
<td>0.689±0.048</td>
<td>Medium</td>
<td>Submetacentric</td>
</tr>
<tr>
<td>17*</td>
<td>0.344</td>
<td>0.463</td>
<td>0.806</td>
<td>0.017±0.002</td>
<td>0.613±0.031</td>
<td>Small</td>
<td>Submetacentric</td>
</tr>
<tr>
<td>18</td>
<td>0.371</td>
<td>0.433</td>
<td>0.804</td>
<td>0.017±0.002</td>
<td>0.540±0.022</td>
<td>Small</td>
<td>Metacentric</td>
</tr>
<tr>
<td>19</td>
<td>0.320</td>
<td>0.393</td>
<td>0.713</td>
<td>0.015±0.002</td>
<td>0.554±0.034</td>
<td>Small</td>
<td>Metacentric</td>
</tr>
<tr>
<td>20</td>
<td>0.234</td>
<td>0.435</td>
<td>0.669</td>
<td>0.014±0.002</td>
<td>0.654±0.029</td>
<td>Small</td>
<td>Submetacentric</td>
</tr>
<tr>
<td>21</td>
<td>0.138</td>
<td>0.470</td>
<td>0.608</td>
<td>0.013±0.001</td>
<td>0.766±0.063</td>
<td>Small</td>
<td>Acrocentric</td>
</tr>
<tr>
<td>X</td>
<td>0.511</td>
<td>0.811</td>
<td>1.323</td>
<td>0.028±0.002</td>
<td>0.615±0.035</td>
<td>Large</td>
<td>Submetacentric</td>
</tr>
<tr>
<td>Y</td>
<td>0.070</td>
<td>0.080</td>
<td>0.150</td>
<td>0.005±0.001</td>
<td>0.607±0.034</td>
<td>Small</td>
<td>Submetacentric</td>
</tr>
</tbody>
</table>

Remark: *=satellite chromosome (nucleolar organizer region, NOR).
karyotype formula for *P. femoralis* could be deduced as: 2n (diploid) 44 = L_{20}^{m} + L_{8}^{sm} + L_{2}^{a} + M_{2}^{sm} + S_{4}^{m} + S_{4}^{sm} + S_{2}^{a} + sex chromosomes.

**GTG-banding and high resolution patterns**

GTG-banding revealed that the number of bands on one set of haploid chromosomes, which includes autosomes and X and Y chromosomes, is 264 bands (Figs. 4 and 5). The number of bands in one set of prometaphase haploid chromosomes identified by the high-resolution technique is 332 bands (Figs. 6 and 7). The GTG-banding and high-resolution techniques provide clear chromosome bands that are dark for heterochromatin and light for euchromatin. The level of GTG-banding and high-resolution techniques (the band numbers) is defined visibly in a haploid set composed of autosomes and the X and Y chromosomes. The haploid set of *P. femoralis* was determined to consist of 21 autosomes including the X and Y chromosomes. However, some chromosomes were not clearly identified because some bands had variations. As above the chromosome band scoring is represent by approximate band that appear.

In addition, a pair of the long arm near the centromere of chromosome pair 17 showed clearly observable NORs. This is the first report of natural polymorphism of NORs in *P. femoralis*. The result showed a heteromorphism in two males and one female with different sizes of NORs of
chromosome pair 17 (Fig. 8). The numbering of regions and bands within the regions follows the International System for Human Cytogenetic Nomenclature (ISCN 1978). Landmarks have been identified and the chromosomes divided into regions. To define a band, the chromosome number, the arm symbol, the region number, the band number, and the sub-band number within that region must be quoted. For example, 2q23.3 indicates chromosome number 2, long arm, region 2, band 3 and sub-band 3.

Discussion

The chromosome number of *P. femoralis* is $2n=44$ and the NF of both sexes is 88. This result agrees with the chromosome characteristics of others in the subfamily Colobinae in Thailand. *T. cristatus* (Hsu and Benirschke 1970, Ponsà et al. 1983, Bigoni et al. 1997a), *T. phayrei* (Nie et al. 1998), and *T. obscures* (Chiarelli 1963, Hsu and Benirschke 1971, Sangpakdee et al. 2008) all have the same chromosome number of $2n=44$ and most have NF=88. In previous studies, the diploid number of both African (genus *Colobus*) and Asian (genus *Presbytis*) colobines was found to be $2n=44$ and most have NF=88 (Chiarelli 1963, Ushima et al. 1964).

The autosomes of *P. femoralis* consist of 24 metacentric, 14 submetacentric, and 4 acrocentric chromosomes. A previous study of the subfamily Colobinae revealed that all chromosomes can be divided into metacentric or submetacentric chromosomes according to their centromeric index, except for those of the Asian langurs, which have a pair of small acrocentric chromosomes that are found to be metacentric chromosomes in the African colobines (Nie et al. 1998). Interestingly, our present study shows four acrocentric chromosomes (two pairs) instead of the two acrocentric chromosomes (one pair) found in Asian langurs according to previous reports (Chiarelli 1963, Hsu and Benirschke 1970, 1971, Ponsà et al. 1983, Bigoni et al. 1997a, Nie et al. 1998).
The X chromosome is a large submetacentric chromosome and the Y chromosome is the smallest submetacentric chromosome. In comparison with the subfamily Colobinae in Thailand, the X and Y chromosomes of *T. cristatus*, *T. phayrei*, and *T. obscures* are submetacentric chromosomes (Hsu and Benirschke 1970, Ponsà et al. 1983, Bigoni et al. 1997a, Nie et al. 1998, Sangpakdee et al. 2008).

In addition, a pair of the long arm near the centromere of chromosome 17 showed clearly observable NORs. The previous study among species of the subfamily Colobinae demonstrated that *P. obscurus*, *T. phayrei*, *T. cristatus*, *S. francoisi* and *Pygatrix nemaeus* have NORs on chromosome pair 21 (Chiarelli 1963, Hsu and Benirschke 1970, 1971, Ponsà et al. 1983, Nie et al. 1998), while the study by Bigoni et al. (1997b) reported that *Colobus guereza* has NORs on chromosome pair 15. The result showed a heteromorphism in *P. femoralis* for both males and female with a difference size of NORs. That is consistent with the reports of Warburton et al. (1975), Van Tuinen et al. (1999) and Tanomtong et al. (2010a) that found different size polymorphisms of NORs in the white-hand gibbon (*Hylobates lar*), moloch gibbon (*H. moloch*), and dark-hand gibbon (*H. agilis*), respectively. Meanwhile Tanomtong et al. (2010b) found the
heteromorphism of NORs (13a13b) in *H. lar*. NORs are composed of high amounts of rDNA, protein and RNA. Ribosomal DNA functions in the 28S and 18S rRNA synthesis in mammals. The rDNA function in rRNA synthesis, so the increase or decrease in size of NORs in *P. femoralis* might influence the gene in protein synthesis (Campiranont 2003). However, Tantravahi *et al.* (1976) and Miller *et al.* (1977) reported that there are NORs on human chromosomes 13, 14, 15, 21 and 22, and that human satellite chromosomes have polymorphism as determined by the NOR-banding technique.

The structure, number and morphology of NORs may be specific to populations, species and subspecies. NORs are frequently used to compare variations, as well as to identify and explain specifications. Changes in chromosome number and structure can alter the number and structure of NORs. Robertsonian translocations may cause losses of NOR. Species that have limited gene exchange due to geographical isolation have elevated karyotype and NOR variety. Therefore, different karyotypes are found even in small but isolated populations of these species. The use of NORs in explaining kinships depends to a large extent on the uniformity of this characteristic and on the degree of variety within a taxon (Yüksel and Gaffaroğlu 2008).

From the GTG-banding technique, the number of bands on one set of haploid chromosomes is 264, and from the high-resolution technique, the number of bands in one set of prometaphase haploid chromosomes is 332; each chromosome pair could be clearly differentiated. In comparison, the study of humans and apes by Yunis and Prakash (1982) reported that the chromosome band number from the high-resolution technique of prometaphase chromosomes is over 1,000 bands per haploid set. The high-resolution banding techniques provide the possibilities to detect chromosome breaks and rearrangement events within major bands. The developments in cell culture and banding techniques have been extremely fast; ten years after the Paris Conference in 1971, Yunis (1981) published the haploid human karyotype at the 1,700-band stage. For other mammals, the
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development was slower, but recently, good results have narrowed the gap between human and
animal cytogenetics. However, the increase of bands in a given karyotype is not an aim by itself,
but a research tool, so the value of a high band level is reduced if band quality is sacrificed in the
process. When high-resolution banding is combined with other chromosomal techniques, specific
sites on the chromosomes can be detected and precisely localized. The high-resolution banding
 technique has been extremely valuable, especially for the localization of single copy genes and
specific breakpoints (Rønne 1991).

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Kaen University, for their assistance.

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homology between human and silvered leaf monkey chromosomes, reveals reciprocal translocations between
chromosomes homologous to human Y;5, 1/9 and 6/16 and delineates an X1X2Y1Y2/X1X2X1X2 sex-chromosome

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