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พันธุศาสตร์เซลล์ของค่างแว่นถิ่นใต้และค่างเทาในประเทศไทย ด้วยการย้อมแถบสีแบบซี

Cytogenetics of Dusky Langur (*Trachypithecus obscurus*) and Silvered Langur (*T. cristatus*) in Thailand by C-banding

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บทคัดย่อ

การศึกษาพันธุศาสตร์เซลล์ของค่างแว่นถิ่นใต้และค่างเทาในประเทศไทยใช้ตัวอย่างจากสวนสัตว์นครราชสีมา เตรียมโครโมโซมด้วยวิธีการเพาะเลี้ยงเซลล์เม็ดเลือดขาว เก็บเกี่ยวเซลล์ด้วยเทคนิคโคลชิซิน-ไฮโพโทนิค-ฟิกเซชั่น-แอร์ดรายอิ้ง ย้อมสีโครโมโซมแถบสีแบบซี ผลการศึกษาพบว่า ค่างแว่นถิ่นใต้และค่างเทามีจำนวนโครโมโซมดิพลอยด์เท่ากับ 44 แท่ง โครโมโซมของค่างแว่นถิ่นใต้ติดสีย้อมที่บริเวณเซนโทรเมียร์และปลายทีโลเมียร์เกือบทุกคู่รวมทั้งโครโมโซมเพศเอ็กซ์และวาย ยกเว้นคู่ที่ 4 18 ที่ติดเฉพาะบริเวณเซนโทรเมียร์ และคู่ที่ 20 ติดเฉพาะบริเวณทีโลเมียร์ ค่างเทาไม่สามารถเก็บผลการทดลองได้ เนื่องจากมีจำนวนเซลล์น้อย จึงนำมาจัดทำเป็นอิดิโอแกรมไม่ได้

ABSTRACT

Cytogenetics of *Trachypithecus obscurus* and *T. cristatus* from Nakhon Ratchasima Zoo were studied. Blood samples were collected and subjected to lymphocyte cultures harvested by colchicine-hypotonic-fixation-air-drying technique followed by C-banding. The results showed that *T. obscurus* and *T. cristatus* have chromosome number 2*n* = 44. Almost all of chromosomes of *T. obscurus* were stained at the centromere, and the apical of telomere including X, Y chromosomes, except 4 and 18, which were stained at the centromere. In addition, the chromosome 20 was stained at the telomere. Due to the low number of cells, we cannot draw the idiogram of the *T. cristatus*.

คำสำคัญ: พันธุศาสตร์เซลล์ แถบสีแบบซี อิดิโอแกรม

Keywords: Cytogenetics, C-banding, Idiogram

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INTRODUCTION

Monkeys are descendants of plesiadapiforms, or tree-dwelling mammals. Since primates spend much of their time in trees, their brains, hands, and feet are well coordinated. This is thought to have had a significant impact on how human evolved (Lertcharnrit, 2007; Laohachinda, 1996). There are currently 232 species of primates in 60 genera across the globe (Wilson and Cole, 2000).

In Thailand, there are three families, five genera, and thirteen species of primates (Lekagul and McNeely, 1977). First, family Loridae consists of only one species: the slow loris. Second, family Cercopitecidae has two subfamily Cercopithecinae, which contains only one genus, *Macaca*. The subfamily Colobinae contains only two genera, with four species found in the genus *Presbytis*, including the Banded langur (*Presbytis femoralis*) and the genus *Trachypithecus*, which contains three species: the Phayre's leaf-monkey (*Trachypithecus phayrei*), the Dusky langur (*Trachypithecus obscurus*), and the Silvered langur (*Trachypithecus cristatus*). Finally, family Hylobatidae, which are gibbons that are reportedly scattered throughout all regions of Thailand. Three species were found including white-handed gibbon, agile gibbon or dark-handed gibbon, and pileated gibbon (Kanwatthanakit, 2000; Lekagul and McNeely, 1977). According to the Wildlife Conservation and Protection Act B.E. 2535 (A.D. 1992), the wildlife species recognized as protected are indicated in Figure 1. The International Union for Conservation of Nature (IUCN) categorises langurs as a low-risk but vulnerable species. The Convention on International Trade in Endangered Species (CITES) lists all langur species under account number 2 or Appendix II. This category includes species that are not necessarily facing extinction but require trade regulation to prevent activities that could harm their survival (Parr, 2003).

C-banding staining is useful in identifying the location of constitutive heterochromatin and the position of DNA with repeated sequences. Chromosome strip dyeing is useful for matching chromosomes. It tells of different chromosomal abnormalities and helps to study the evolution of living organisms (Kampiranont, 2003). Myers and Shafer (1979) found that the absence of identical chromosomes suggests that the arrangement of chromosomal structures plays an important role in the genetic inheritance of tailless monkeys. The 1983 study by van Tuinen and Ledbetter on the comparative genetics and evolutionary lines of all 3 gibbons in the subgenus *Hylobatidae* indicates that C-banding results showed abnormal banding, and NORs or satellite chromosomes, were found in chromosomes. Currently, cytogenetics is extensively examined in numerous animals, including langurs. At present, langurs have been discovered to be threatened by both humans and other animals.

As a result, it is important to understand the fundamental genetics of these species in order to maintain the long-term survival of langur species, it is also necessary to use the knowledge that has proven effective in other locations. In the meantime, the objective of the study was to examine the C-banding dyeing techniques used for both langur species in Thailand in order to provide a basis for genetic investigations on various features of langurs.

Figure 1 The four species of langur found in Thailand; Banded langur (*Presbytis femoralis*) (A), Silvered langur (*Trachypithecus cristatus*) (B), Dusky langur (*Trachypithecus obscurus*) (C), Phayre's leaf-monkey (*Trachypithecus phayrei*) (D)

MATERIALS AND METHODS

The tools and methods of operation were divided into 4 stages: sampling, chromosome preparation, C-banding chromosome dyeing, and chromosome inspection by karyotyping and idiograming.

1. Sampling

There were two species of blood samples used in this study, collected from male and female Dusky langurs and Silvered langurs, which were kept at Nakhon Ratchasima Zoo. Using aseptic techniques, the veterinarian collected about 5 milliliters of blood from the jugular vein into 10 milliliter vacuum tubes containing heparin to prevent blood clotting. The blood samples were kept cool in ice flasks throughout the laboratory process.

2. Preparation of chromosomes

Chromosome preparation was adapted from Kampiranont (2003) and Rooney (2001) with the following preparation procedures:

2.1 Cell culture

Prepared stock culture, the RPMI 1640 medium add phytohaemagglutinin (PHA) for cell division stimulants were kept in blood culture bottles of 10 ml each. A blood sample of 0.5 ml was dropped into a medium bottle and well mixed. The culture bottle was loosely capped, incubated at 37 ºC in 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 minutes.

2.2 Cell harvest

The blood sample mixture was centrifuged at 1,200 rpm for 10 minutes and the supernatant was discarded. Ten ml of hypotonic solution (0.075 M KCl) was applied to the pellet and the mixture was incubated for 30 minutes. The supernatant containing KCl was discarded after centrifugation again at 1,200 rpm for 10 minutes. Cells were fixed by fresh cold fixative (methanol: glacial acetic acid in a ratio of 3: 1) gradually added up to 8 ml before centrifuging again at 1,200 rpm, and the supernatant was discarded. The fixative 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.

3. C-banding technique

Fifty ml of 2X standard saline citrate (SCC) was put in a staining bowl and heat in the water bath before use. The slide was well dried and then soaked in HCl concentration 0.2 N for 10 - 15 minutes; rinsed with distilled water twice; then the slide was soaked in working $BaOH_2$ solution for 30 minutes via a quick slide in a 2X SSC solution and passed quickly in 95% ethanol. The slide was dried and placed it in a wet chamber, which contains a 2X SSC solution as a moisturizer on the slide. A few drops of 2X SSC solution was dripped with a coverslip and incubated it in the wet chamber in a 60 ºC incubator for 1 hour afterwards. The slide was rinsed with distilled water and then stained with 5% Giemsa's solution for 30 minutes.

4. Chromosome investigation

Chromosome checking, karyotyping and idiograming were adapted from Chaiyasut' s (1989) methods.

4.1 Chromosome checking

Twenty clearly observable and well-distributed chromosomes of each male and and female langur were selected. There was no overlap, and the total number of chromosomes was studied under a microscope, photographing chromosomes in the metaphase phase. The chromosome were analyzed by determining the length of the arms, short arms (Ls), arm length, long arms (Ll), and then calculating the length of each chromosome (Ll+Ls). The relative length (RL), the centromeric index (CI) and the standard deviation (SD) of RL and CI were also computed to classify the type and size of chromosomes according to the resulting value of all parameters used in karyotyping and idiograming.

4.2 Karyotyping and idiograming

Karyotype was a detailed study of all chromosomes in each organism. Twenty cells were clearly studied for both the number of chromosomes and the shape of the chromosome. The karyotype used chromosomes from a metaphase of only one cell to represent that organism.

RESULTS AND DISCUSSION

The study of the cytogenetics of langurs was conducted using animal samples from Nakhon Ratchasima Zoo. A total of two samples including 1 male and 1 female were studied for each species.

The Dusky langur had the number of chromosomes 2*n* (diploid) = 44, which can be determined through karyotyping and idiograming (Figures 2, 3, and 4). The chromosomes were attached to the dye only at the centromere area, namely pairs 4 and 18 chromosomes, attaching dyes around centromeres and telomeres on the short side chromosome arm, pairs 1, 2, 3, 5, 6, and 7, and attaching dyes around centromeres and telomeres on the long side chromosome arm, pairs 10, 21, and sex chromosomes X and Y. The C-banding dyed regions at centromeres and telomeres on short and long chromosome arms pairs were: 8, 9, 11, 12, 13, 14, 15, 16, 17, and 19 chromosomes, but only centromeres on the pair of 20th chromosomes.

The Silvered langur had the number of chromosomes 2*n* = 44, which can be karyotyped (Figures 5 and 6). However, the idiogram cannot be rearranged due to the limited number of cells. Clear metaphase and karyotype images, as well as a large number of cells, are necessary for achieving precise and accurate experimental results.

In the investigation of the satellite chromosomes, or marked chromosomes, we found that the Dusky langur (*T. obscurus*) and the Silvered langur (*T. cristatus*) had one pair on the autosome pair of 17th chromosomes (Figures 2, 3, 5, and 6). The result showed a heteromorphism in *T. obscurus* and *T. cristatus* for both males and females, with a difference in the size of the satellite chromosomes. Meanwhile, heteromorphisms in satellite chromosomes were composed of high amounts of rDNA, protein, and RNA. The rDNA functions in rRNA synthesis; thus, an increase or decrease in the size of satellite chromosomes in *T. obscurus* and *T. cristatus* might influence the gene in protein synthesis (Campiranont 2003). From the search, it was found that satellite chromosomes on the autosome pair of 17th chromosomes could also be found in two other langurs, *P. femoralis* and *T. phayrei,* for both males and females, with a difference in the size of the satellite chromosomes (Tanomtong *et al.*, 2014; Pinthong *et al.*, 2018).

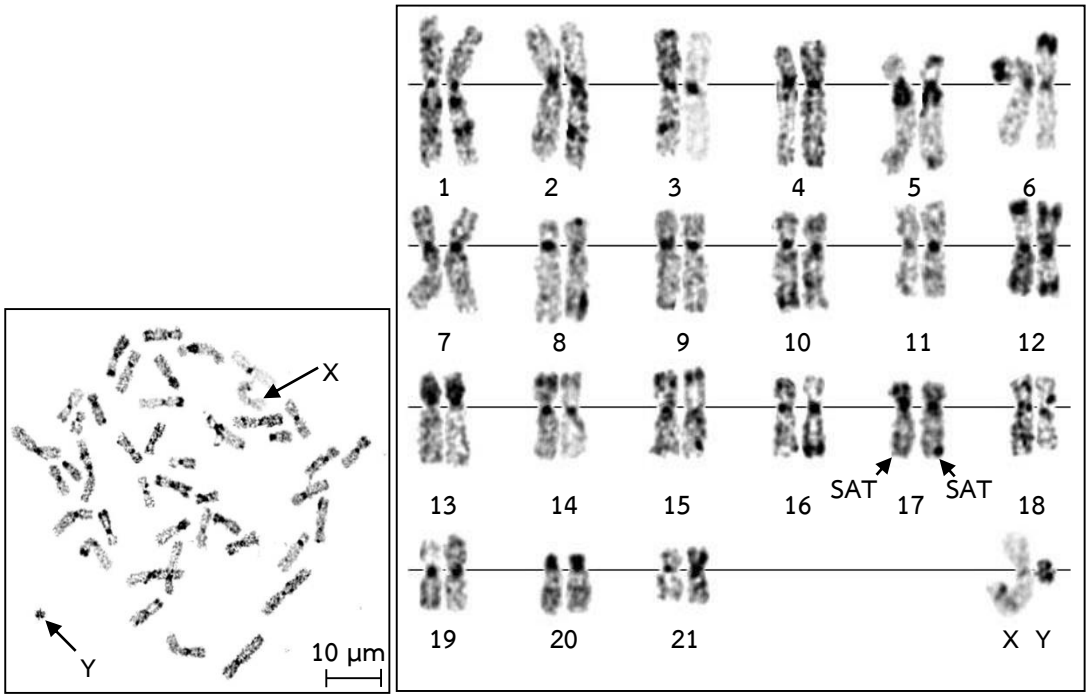

Figure 2 Metaphase chromosome plate and karyotype of male Dusky langur (*T. obscurus*), 2*n* = 44 by C-banding chromosome dyeing technique. Arrows indicate pair 17 at satellite chromosomes (scale bar = 10 µm).

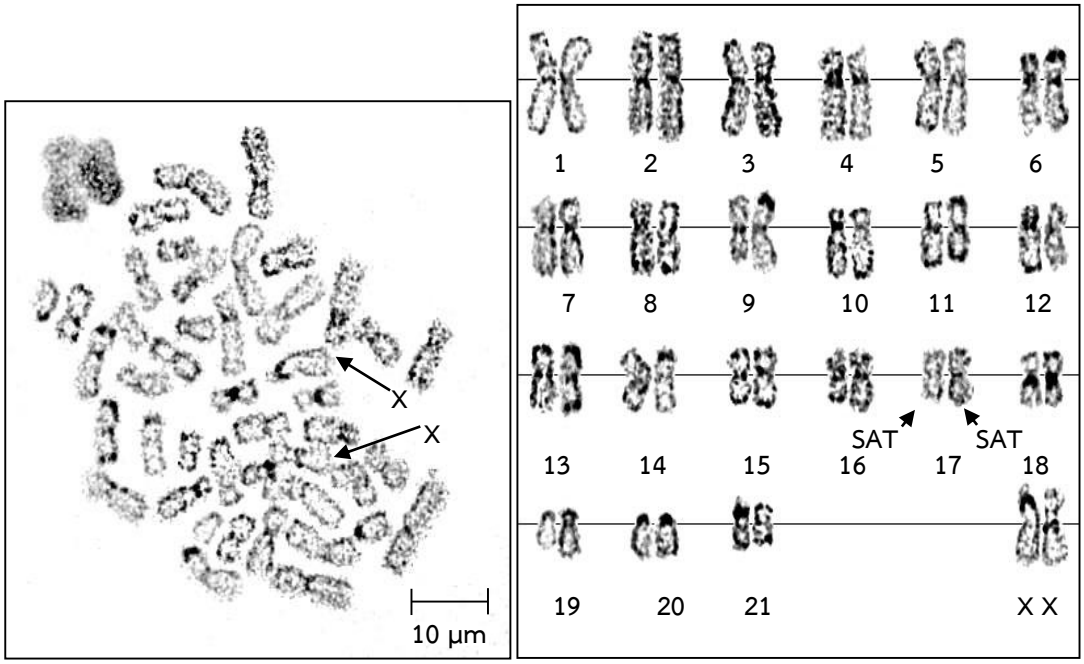

Figure 3 Metaphase chromosome plate and karyotype of female Dusky langur (*T. obscurus*), 2*n* = 44 by C-banding chromosome dyeing technique. Arrows indicate pair 17 at satellite chromosomes (scale bar = 10 µm).

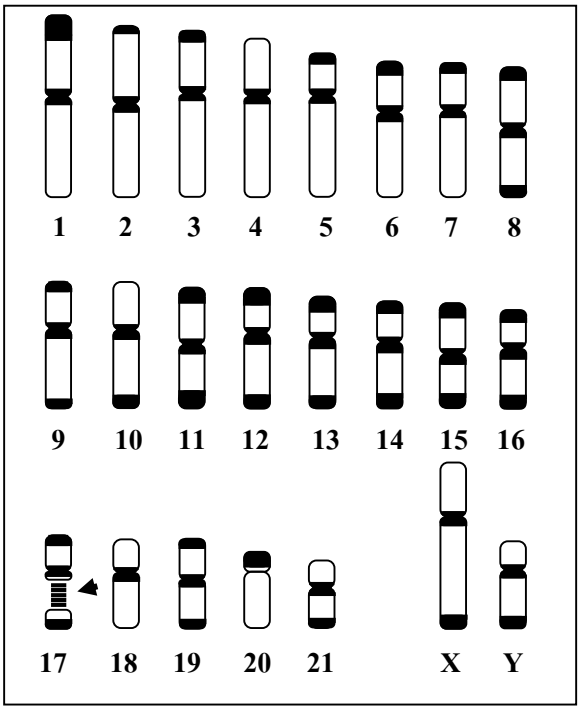

Figure 4 Idiogram of Dusky langur (*T. obscurus*, 2*n* = 44) with C-banding chromosome dyeing technique. Arrow indicates pair 17 at satellite chromosome.

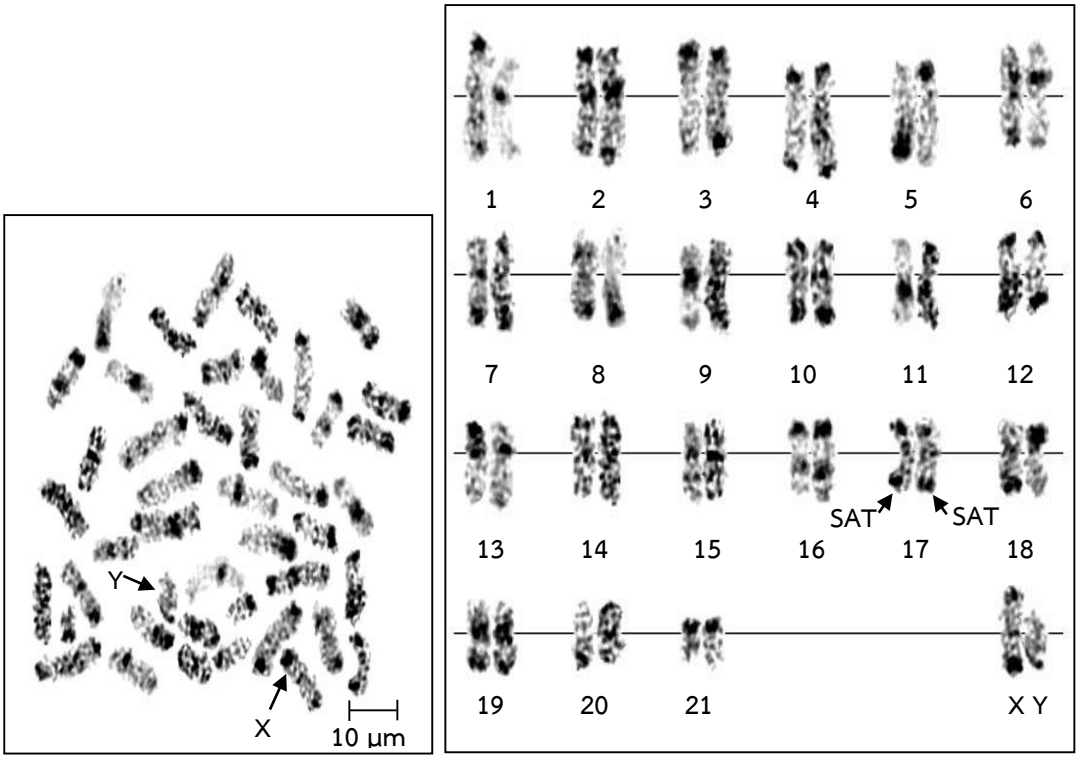

Figure 5 Metaphase chromosome plate and karyotype of male Silvered langur (*T. cristatus*), 2*n* = 44 by C-banding chromosome dyeing technique. Arrows indicate pair 17 at satellite chromosomes (scale bar = 10 µm).

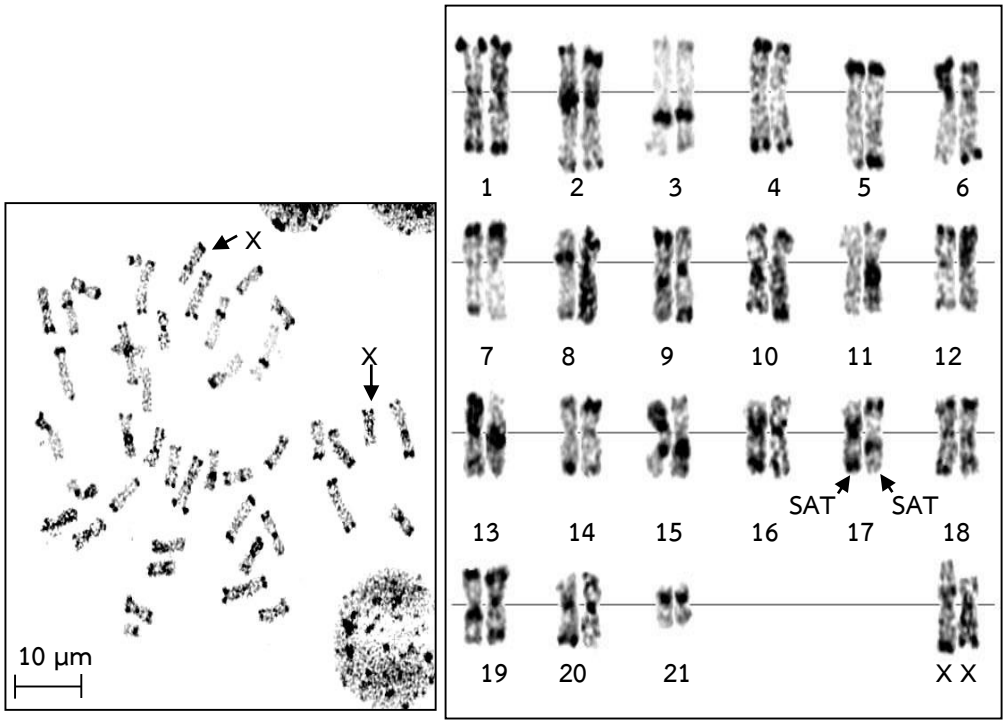

Figure 6 Metaphase chromosome plate and karyotype of female Silvered langur (*T. cristatus*), 2*n* = 44 by C-banding chromosome dyeing technique. Arrows indicate pair 17 at satellite chromosomes (scale bar = 10 µm).

A study of the cytogenetics from C-banding chromosomes of two langurs, the Dusky langur and the Silvered langur, demonstrated that the chromosome total number was 2*n* = 44. After collecting blood samples and cultivating lymphocytes and harvested cells, we found that both male and female Dusky langurs and Silvered langurs had sediment lymphocytes. However, the cell sediment of Silvered langurs was lower than Dusky langurs, possibly due to cell culture problems. Under a microscope, many cells of the Silvered langurs had more than 44 chromosomes and they were overlapping. Therefore, there were not enough cells for idiograming. Both male and female Dusky langur and Silvered langur cells can be karyotyped. It can only be used to idiogram Dusky langur since Silvered langur had too many chromosomes, insufficient cells, and a weak dye staining. Incomplete metaphase cells may cause this issue.

According to a study based on the C-banding chromosome dyeing technique in Dusky langur, most chromosomes were colored around centromeres, and some chromosomes were colored around centromeres and telomeres. These were consistent with the findings of Wijayanto *et al*. (2005), who examined the primate chromosomes of the genera *Hylobates* and *Symphalangus* using the C-banding technique. *Hylobates* chromosomes were found to be colored around centromeres. The chromosomes of *Symphalangus* were colored around centromeres and telomeres. In accordance with a study by Hirai (2001) that studied chromosomes with the C-banding chromosome dyeing technique in primates including humans, bonobo, gorillas, and siamang, human chromosomes were found to be attached to every centromere, except for chromosomes 1, 9, and 16, and the long arms of chromosome Y had larger color

stripes than all chromosomes. From the C-banding chromosome dyeing technique, chromosomes were colored around centromeres, telomeres, and on the short arms of pairs of chromosomes 14, 15, 17, 22, and 23. Differences from gorilla chromosomes were colored around the paracentromeric area in some chromosomes; the telomeres of the Y chromosome colored large bands in the middle of the long arm; and they also found heteromorphism in chromosome pair 18. In addition, in siamang, chromosomes were colored at both ends of all chromosomes, except for the 12 and 21 pairs of chromosomes; the short arm of the 21 chromosome rod was NORs, and the Y chromosome stain was the same color as the Y chromosome of the bonobo. Also corresponded to Fukushi and Ushiki (2005) showed the structure of C-banded human metaphase chromosomes and revealed that only the centromeres of human chromosomes were stained. Chromosome staining with the C-banding revealed the constitutive heterochromatin in male Dusky langur chromosomes 3, 10, 16, 18, and 21. They were caused by chromosomal changes in the form of the addition of heterochromatin to karyotypes, which means they were chromosomal abnormalities.

Even though they are the same organism, mammalian centromeres may have different sizes or numbers and have heterochromatin features. They have primarily alpha DNA with a double resonance of 5 – 170 base pairs. The centromere was polymorphic. The centromere's shape was also influenced by chromatin folding and binding proteins. Chromosome abnormalities may have several centromeres, but only one will be active. Cell division and genetic inheritance proceed normally because the other region is inhibited and finally fades. Most studies focused on human Y chromosomes. Alpha-type DNA with redundancy of 200 and 300 kilobases, part of the short arm, was located in the centromere area. They aid each other during cell division. Thus, centromere formation and function depend on alpha DNA with duplicate base sequences; if alpha DNA was absent, it could stop chromosomal function (Kangwanpong, 2003).

CONCLUSIONS

According to C-banding chromosome dyeing of Dusky langur and Silvered langur, the chromosomes of Dusky langur chromosomes were stained with specific dyes in the centromere region, including the 4 and 18 pairs of chromosomes. The dye is visible in the centromere and telomere on the short arm chromosome; pair the 1, 2, 3, 5, 6, and 7 chromosomes. It is also observable around the centromere and telomere on the long arm chromosome, including the 10, 21, X, and Y chromosomes. The dye is evident in the centromere and telomere of chromosomes with short and long arms, including the 8, 9, 11, 12, 13, 14, 15, 16, 17, and 19 pairs, but only on the centromeres of the 20^{th} pair. The number of chromosomes was $2n = 44$. Additionally, the chromosome 17 was the satellite chromosomes. Due to the limited number of cells, Silvered langur cannot capture experimental data. This renders the organization of an ideogram impossible.

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