MATERIAL AND METHODS

Sampling Locations

Most of the samples were obtained from captive gibbon near their original habitat. Blood samplings were taken with the permission of the Indonesian Research Authority/Lembaga Ilmu Pengetahuan Indonesia (LIPI) and Indonesian Forestry Protection and Nature Conservation/Perlindungan Hutan dan Pelestarian Alam (PHKA), with the assistance from the rangers of the local Natural Resources Conservation Office/Balai Konservasi Sumber Daya Alam (BKSDA) in West Sumatra, Central Kalimantan, and South Kalimantan.

Sumatra and Kalimantan were selected as the locations of blood sampling of gibbons since both of the islands are the natural habitat of agile gibbons. H. agilis albibarbis (Kalimantan); and H. agilis agilis and H. agilis unguco (Sumatra) belong to the same species H. agilis. Recently, a subspecies H. agilis albibarbis was promoted to be separated as a species rank (Groves 2001).

Methods

Blood sampling

Blood collection was conducted as described before (Hirai et al. 1998). Whole blood (less than 1 ml/kg) from gibbons was collected using heparinized syringes under anesthetized conditions with ketamine hydrochloride. The methods of anesthesia (7.5 mg ketamine/kg body weight by an intramuscularly injection) and blood sampling (2 ml/kg body weight) were in accordance with the guidelines for the care and use of primates of the Primate Research Institute, Kyoto University; KUPRI; Primate Research Institute, Kyoto University 2002 and the Primate Research Center, Bogor Agricultural University 2003. Blood samples were cultured at the Primate Research Center; Bogor Agricultural University, Bogor, Indonesia, and chromosome...
preparations were made after 70 h culture, as previously described (Hirai et al. 2003).

The fixed cell preparations and purified DNA were transferred to KUPRI with the permission of CITES (Export No. 02830/IV/SATS-LN/2003 and 08556/IV/SATS-LN/2003; Import No. 5020/14/0002 and 5020/15/0001) for chromosome analysis.
Figure 1. Map of West Sumatera and Central Kalimantan. Blue stars are places where the samples were collected in the origin habitat. West Sumatera, *H. agilis agilis* (upper), Central Kalimantan (bellow), *H. agilis albibarbis*, and South Kalimantan, *H. muelleri*

Chromosome preparation

Chromosome preparation was conducted at the Laboratory of Virology, Primate Research Center, Bogor Agricultural University. The technique was adopted from Hirai *et al.* (1998). One milliliter of whole blood was cultured in 9ml PRMI 1640 medium containing the following agents: 20% FCS, mitogen (10 µg/ml phytohemaglutinin), 50-ug/ml streptomycin, and 50 units penicillin. The culture was incubated in 5% CO₂ incubator, 37°C for 70 hr. The culture was treated with 50 ug/ml colcemid 30 minutes before harvest. Harvested cells were centrifuged at 1400 r.p.m. for 5 min, then the pellets were incubated as a mixture (total 4 ml) containing 3.5 ml of 0.56% KCl at room temperature for 20 min. The cell suspension were fixed with 7 ml of ethanol-acetic acid (3:1) for 10 min at room temperature, and then centrifuged again to obtain cell pellets. After once more washing and centrifugation, the cell pellets were preserved with 1 ml of fixative in a freezer at –20°C until the observation.
Laboratory techniques

C-banding and DAPI staining were performed following the standard procedure adopted in the Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University (Hirai 2001). DAPI staining was done prior to the C-banding staining and proved very useful to clarify the C-banding pattern of each chromosome.

**DAPI-C band sequential staining**

Each chromosome was first identified using DAPI (4,6-diamidino-2-phenylindole) staining prior to C-banding in order to describe C-band traits of all chromosomes. DAPI fluorochrome has a higher affinity with A+T-rich DNA than with G+C-rich DNA (reviewed by Sumner 1990), so that it can be used to define the C-bands. Brighter fluorescence is positive DAPI and contains A+T rich DNA, while dull band is negative DAPI, G+C rich DNA.

DAPI staining was done by denaturing slides in 2xSSC pH 12.5 for 4.5 min followed by 70% and 99.5% alcohol dehydration for 5 min each, and covering with anti-fade solution containing DAPI (50 ng/mL). A cover slip was attached after drying and immersing in BI buffer (0.1 mol/L sodium bicarbonate and 0.1 % IGEPAL (Sigma) for 5 min.

DAPI bands were observed using a Zeiss Axiophot fluorescence microscope and images were saved into a computer with a CCD camera system. After saving data, the cover slip of the DAPI-stained slide preparation was removed and the anti-fade solution was washed out with running tap water and immersing in distillated water for 1 hr (Hirai, 2001). The slide was treated for C-banding, which is a slightly modified version of a standard technique from Sumner (1972). Briefly, the chromosome preparation was treated with 0.2N HCl for 30 min, soaked in 5% barium hydrochloride at 53°C for 7 min, rinsed in 2xSSC at 53°C for 25 min, and finally stained with 4% Giemsa (Merck) in Sörenssen’s buffer (pH 6.8) at room temperature for 30 min. The chromosome preparation was completely washed out of each reaction solution under running
tap water at the intervals between each step. After drying, the stained slide was covered with a mounting reagent (Malinol) and a cover slip, and the C-banded chromosomes were analyzed for chromosome spreads examined DAPI bands. Treatment temperature 53 °C showed more stable stainability for C-banding after DAPI staining rather than 55°C used for direct C-banding, being also needed to get clearer standard C-band patterns (for the detailed method, see the article text).

**Nomenclature for classifying C-banded chromosomes**

To classify C-banded karyotypes of small apes, we used Imai’s TAM system (Imai 1991; Imai et al. 2001) slightly modified by Hirai et al. (2002), which is very useful for identifying chromosomes with terminal (t), interstitial (i), and pericentromeric (c) C-bands.

![Diagram of C-banding pattern](image)

**Figure 2.** Nomenclature of C-banding pattern according to TAM system (Imai 1991 and Imai et al. 2001), modified by Hirai (Hirai et al. 2002). Imai classify chromosome into four shapes, A, A\(^M\), M\(^A\) and M. To describe the agile gibbon chromosome, which showed i (interstitial), t (terminal), and c (pericentromeric) C-band, the nomenclature system then was modified using term of cit. A slash (/) between superscripts letters is for indicating which arm (short or long) has the cit (Hirai et al. 2002). That is, the left side is in the short arm and the right in the long arm.
Basically, C-banded chromosomes were divided into four types distinguished with chromosome morphology and C-band system: A and $A^M$ (‘A’ chromosome) are chromosomes with the totally heterochromatic short arm and euchromatic long arm, while $M^e$ and M (‘M’ chromosome) are chromosomes with euchromatic short and long arms. Each chromosome type can be characterized in more detail in terms of ‘c’, ‘i’, and ‘t’ band. A slash (/) between superscripts letters is for indicating which arm (short or long) has the cite (Hirai et al. 2002). That is, the left side is in the short arm and the right side is in the long arm.

**PRINS localization of telomeric DNA**

Telomeric sequences $(T_2 AG_3)_n$ were located using PRINS (primed *in situ*) technique as described by Hirai (2001). Reaction solution was prepared following the manufacturer’s protocol (Boehringer Mannheim, Dig-PRINS reaction kit). The results were observed and saved using a Zeiss Axiophot epifluorescence microscope with a cooled CCD camera system (Photometrics, PXL) connected to computer (G4, Apple Macintosh) running IPLab software (Scanalytics).
**Data analyses**

Chromosome data were analyzed descriptively, while TSPY divergence was confirmed by a genetic distance analysis with micro satellite DNA fragments. The FST value was calculated and a 5000 time permutation test for its significance was performed by AMOVA using data from 195 alleles of 12 micro satellite loci.

**References**


Differentiation and Confirmation of C-heterochromatin Traits Using DAPI/C-band Sequential Staining in Chromosomes of the Agile Gibbon (*Hylobates agilis*) and the Siamang (*Symphalangus syndactylus*)

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Abstract

Wide varieties of staining techniques have been used in chromosome studies and produce characteristic banding patterns of chromosomes that are important clues for research of chromosome evolution. One of those techniques, the DAPI (4′-6-diamidino-2-phenylindole)/C-band sequential staining technique is method of choice for chromosome identification in species and locations of constitutive (C) heterochromatin. The DAPI fluorescence banding technique is simple and is applicable in the limited laboratory facilities as well and does not comparatively damage chromosomes, and if it is combined with C-banding technique, thereby, provides a very useful method to clarifying the heterochromatin traits. The present report uses a C-banding technique with barium hydroxide which stains in particular C-heterochromatin. Application of C-band staining in chromosomes of the agile gibbons, a species of lar-group gibbons, need a slightly modified technique compared to the standard protocol, since the animal has more sensitive stainability for C-banding than other hominoids. Our trials showed that temperature plays a critical role in obtaining good C-bands in the chromosomes of lar-group gibbons. Reducing incubation temperature (from 60 to 53°C) of 5% Ba(OH)₂ for 7 minutes and 2xSSC for 25 minutes followed by soaking in 4% Giemsa solution at room temperature for 30 minutes resulted in more contrast banding than using the standard protocol. Moreover, the DAPI/C-band sequential staining technique revealed that C-heterochromatin of lar-group gibbon and siamang contain G+C and A+T rich DNA segments, respectively.

Key words: DAPI, C-band, heterochromatin, *Hylobates*, *Symphalangus*.
Introduction

Many methods of chromosome staining have so far been established in cytogenetic studies. Chromosomes rearrangements occurring during evolution can be tracked back by chromosome staining, and allow us to determine the break points precisely. One of the useful techniques is C-banding method, detecting constitutive (C-) heterochromatin. Heterochromatin was firstly defined by Heitz (1928) as chromatin that did not decondense at the end of the telophase, but instead remained compact throughout the interphase, and was found to be condensed even at the beginning of the prophase (Sumner 2003). Many methods have been tried to demonstrate C-bands on chromosomes during several decades. For instance, C-band produced by DNase have been used on sectioned material by Yamasaki, however, inconsistencies on C-banding patterns some times occurred using such method, Katho et al. demonstrated heterochromatic bands in chromosomes of the Indian muntjac, which were different from the C-bands shown by the BSG technique, Dev et al. used incubation in formamide solution which were adopted by Marshall to induce C-bands, unfortunately this protocol only worked in the chromosomes of mice, but not in chromosomes of rat, man, or hamster, after trypsin G-banding (Yamasaki 1961; Katho et al. 1974; Dev et al. 1972; Marshall 1975, reviewed in Sumner 1990). To solve the inconsistency of C-bands results, Sumner (1972) introduced the BSG (Barium hydroxide/Saline/Giemsa) method, which has became the standard method for producing C-bands on plant and animal chromosomes (Sumner 1990). However, standard protocol of C-banding, which was applied for staining agile gibbon chromosome in fact did not work well. In the present study, an experiment was conducted to modify the standard technique of C-banding, which allowed to visualize C-band of agile gibbons chromosome more clearly and readable for banding analysis.

Although, chromosome painting is an advanced technique of fluorescence in situ hybridization (FISH) and is also very useful for obtaining a high resolution of chromosome identification, but, such technique requires sophisticated facilities in
more advanced laboratories. The present study was conducted to survey the basic information in chromosomes of small apes that have little been noted so far though actually have informative traits, so that the technique using ordinary facilities were also available. C-bands have little been noted in small apes so far, but actually were useful for detecting a hybrid between different genera (Myers & Shaver 1979; Pellicciari et al. 1988), a unique whole arm translocation within a species of small apes (Hirai et al. 2003), and important traits of chromosomes of agile gibbons that have not been previously uncovered (Wijayanto et al. 2005 in printing). The DAPI staining technique which was used prior to the C-banding in the present study is useful in identifying chromosomes of mammals. Moreover, because DAPI has high affinity to A+T rich DNA, application of DAPI/C-band sequential staining technique allows us to clarify the DNA content of the heterochromatic region of chromosomes. This report will describe detailed methods that we adopted here to detect C-bands and the molecular traits in agile gibbons and siamang that have different C-band features from each other.

Materials and methods

Materials

Blood samples of forty-four agile gibbons and one siamang were obtained from Indonesia (Sumatra and Kalimantan); the Primate Research Institute, Kyoto University, Japan, provided four samples; and siamang blood samples were kindly provided by the Japan Monkey Center, Japan. Whole blood samples (0.7-0.8 mL) were collected with a heparinized syringe from individual agile gibbon anaesthetized with ketamine hydrochloride and cultured as described by Hirai et al. (2003).

Methods

DAPI-C band sequential staining

a. DAPI Staining

Stock Solutions: 5 M NaOH, 20xSSC, 99.5% ethanol, and DAPI Stock solution.
**Working solutions:** 2xSSC pH 12.5 (5 ml 20xSSC + 500 ul 5M NaOH + 44.5 ml dH₂O); 70% ethanol (35 ml ethanol absolute + 15 ml dH₂O); BI buffer (50 ml 1M NaHCO₃ + 500 ul Igepal + 450 ml dH₂O); DAPI + PI (Dissolve DAPI stock solution and PI in dH₂O and make a final concentration containing 500 ng/ml DAPI 10, and ng/ml PI).

Procedures were done sequentially as follows:

1) denatured slide by placing in 2xSSC pH 12.5 for 4 minutes,
2) dehydrated slides in 70% and 99.5% ethanol for 5 minutes each,
3) dried at room temperature,
4) immersed in BI buffer for 5 minutes,
5) mounted with 20 µl anti-fade solution contained DAPI and PI and a cover slip.

DAPI bands were observed by using a Zeiss Axiophoto fluorescence microscope and saved into a computer with a CCD camera system. The good chromosome spread locations were recorded. After saving data, the cover slip of the DAPI-stained slide preparation was removed and the anti-fade solution was washed out with running tap water and the slide was immersed in distilled water for 30 minutes (in Hirai 2001, 1 hour). The slide was treated for C-banding, which is a slightly modified version of the standard technique (Sumner 1972).

b. C-banding staining after DAPI (Barium-Saline-Giemsa/BSG) were done with solutions: 0.2 N HCl (10 ml 1 N HCl + 40 ml dH₂O); 5% Ba(OH)₂ (2.5 g Ba(OH)₂ + 50 ml dH₂O); 2xSSC (0.3 M sodium containing 0.03 M trisodium citrate); 4% Giemsa (2 ml Giemsa + 48 ml Sörenssen’s buffer pH 6.8).
Sequential procedures:

1) removed cover slip of DAPI-stained slides, wash out anti-fade solution with running tap water and immerse the slides in dH₂O for 30 minutes,
2) soaked in 0.2 N HCl for 30 minutes,
3) washed vigorously in running tap water,
4) treated in 5% Ba(OH)₂ 53°C for 7 minutes,
5) washed vigorously in running tap water,
6) soaked in 2xSSC 53°C for 5 minutes,
7) stained by 4% Giemsa solution for 30 minutes,
8) washed vigorously in tap water,
9) dried the slides in a 37°C incubator or at room temperature over night,
10) covered using Malinol mounting solution and a cover slip,
11) observed chromosome spreads, which have had recorded in the DAPI stained preparation, being able to exactly compare the data of both DAPI-staining and C-banding analyses.

Results and discussion

DAPI staining

The DAPI staining technique that was used in the present study clearly manifested G-like bands on chromosomes of both agile gibbons and siamang. Figures 1 and 2 showed DAPI-stained metaphase spreads of both species. The two pictures demonstrated different features of stain condition between agile gibbon (Figure 1a) and siamang (Figure 2a). That was, most chromosomes of siamang have the brightest fluorescent band at both of the terminal regions, but agile gibbons have no such brighter band on chromosome ends. It was very contrasting.
Figure 1. Chromosomes of agile gibbons. DAPI staining (a) and C-band after DAPI staining (b). Negative DAPI band were expressed as positive C-band (arrowhead) and vice versa (arrow). Scale bar 10 μm

Figure 2. Chromosomes of siamangs. DAPI staining (a) and C-band after DAPI staining (b). Positive DAPI band were also expressed as positive C-band (arrowhead) and vice versa (arrow)
Comparison between DAPI- and C-bands

The standard protocol for BSG (Barium/Saline/Giemsa) C-band staining (e.g., Sumner 1972) was not suitable for staining C-heterochromatin of chromosomes of agile gibbon, though those of siamang showed good results by the standard technique.

![Diagram](image)

Figure 3. Ideogram explains the expression of DAPI and C-band sequential staining in chromosomes of agile gibbons (*Hylobates*) (left) and siamangs (*Symphalangus*) (right). DAPI and C-band between them (arrow) showed opposite traits. *Hylobates* has C-bands of negative DAPI, while *Symphalangus* has C-bands of positive DAPI.

Several techniques of C-banding were tried to find an adequate procedure for detecting C-bands of agile gibbons that seem to be more vague and complicated rather than other primate species. For example, human chromosomes have C-bands (Figure 4b) that can be detected by a standard technique of BSG established by Sumner (1972), and siamang used in the present species also have the same features of C-band as human, being stainable by the standard technique (see Figure 2b). Finally, the present trials uncovered a best technique using treatment of Ba(OH)$_2$ at 53$^\circ$C for 7 minutes. Figure 4c shows the best results observed with the modified technique.
Discussion

The DAPI (4’-6-diamidino-2-phenylindole) staining performed prior to C-banding gave a significant contribution to determining the location of heterochromatic band in the agile gibbon chromosomes, and to detect molecular feature of the bands. DAPI fluorochrome has higher affinity to A+T than to G+C rich DNA (Verma and Babu 1995; Sumner 1990). Therefore, in the present study DAPI/C-band sequential staining was applied to investigate molecular features of C-bands of agile gibbons and siamang. Consequently, C-bands of agile gibbons all accorded with dull (negative) bands of DAPI-staining, meaning that the bands were G+C-rich. On the other hand, terminal C-bands of siamang corresponded with bright (positive) bands of DAPI-staining, meaning that the bands were A+T-rich.

Using the procedure mentioned above, it was found that C-bands of agile gibbons contained G-C rich, while those of siamang had A-T rich DNA. Moreover, the banding analysis showed the C-band pattern of agile gibbons is specific for each chromosome and thus very useful for identification of chromosomes. If the original standard technique of BSG method were used here, C-bands of agile gibbons would have remained unreadable as shown in Figure 4a. As the standard method, BSG method used Ba(OH)₂ treatment (Sumner 1972) instead of NaOH to denature DNA became a good method for obtaining C-band, since it provided an advantage which facilitates better control of the denaturation process than NaOH (Verma and Babu 1995).

Ba(OH)₂ is a relatively milder alkali than NaOH, slide preparations must be subjected to a longer treatment with Ba(OH)₂ to denature chromosomal DNA, and thus the denaturation process is easier than others. Nevertheless, temperature and time of treatment in Ba(OH)₂ is critical to obtain higher quality C-bands. Generally, the duration of treatment depends on the age of slide preparations and the origin of material. However, the case in the present study seems to be related to the essential
characteristics of C-bands of agile gibbons, though the precise mechanism is unknown. That is, lower temperature (from 55°C to 53°C) and shorter time (7 minutes and 25 minutes, respectively) in 5% Ba(OH)₂ and 2xSSC treatment were resulted better chromosome morphology and band quality (Figure 4c), although still not as good as human chromosome (Figure 4b).

Figure 4. C-banding after DAPI-staining. Standard method of BSG (Sumner 1970) produced vague bands in agile gibbon (a), but clear band in human chromosome (b). Modified protocol of BSG applied to *Hylobates* chromosomes shows interstitial, terminal, and paracentric heterochromatic bands (c). Scale bar 10 µm.
Figure 5. Schematic diagram of C-banding pattern of *Hylobates* chromosomes (left) with interstitial, terminal, and paracentric heterochromatic bands and *Symphalangus* (right) with bigger bands at the distal end of biarmed chromosome. Arabic numbers below are chromosome number. Chromosome 12 and 21 of siamang has unique C-bands patterns. As shown, chromosome 12 has telomeric in the short arm and interstitial C-band in the long arm, and chromosome 21 has negative short arm with a centromeric and a telomeric C-bands in the long arm.

Treatment temperature 53 °C also showed more stable stained ability for C-banding after DAPI staining rather than 55 °C used for direct C-banding. This could

<table>
<thead>
<tr>
<th>Reaction solution</th>
<th>Modification method (in the present study)</th>
<th>Standard method (Sumner 1972)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2N HCl</td>
<td>30 minutes at room temperature</td>
<td>1 hr at room temperature</td>
</tr>
<tr>
<td>5% BaOH</td>
<td>53 °C, 7 minutes</td>
<td>50 °C, 5-15 minutes</td>
</tr>
<tr>
<td>2xSSC</td>
<td>53 °C, 25 minutes</td>
<td>60 °C, 1 hr</td>
</tr>
<tr>
<td>Giemsa</td>
<td>4% Giemsa in Sorenssen’s buffer pH 6.8, 30 minutes</td>
<td>2% Gurr’s Giemsa in buffer pH 6.8, 90 minutes</td>
</tr>
</tbody>
</table>

Table 1. Comparation between modified C-band and the standard staining method of Sumner (1972) used in the present study
be related to use of 2.5 pH NaOH 2xSSC solution for DAPI-staining. As with other C band staining methods, freshly prepared slides are usually unsuitable for C-banding, because the chromosome may be too sensitive to withstand harsh treatment, and they thus lose their morphology, becoming fuzzy and hollow in appearance. That is the reason, why the slide preparations have to be aged for either one week or ten days at room temperature or for 2 to 3 days at 50 to 60°C (Verma and Babu 1995). Slide preparations were kept in a 37°C incubator for four to seven days.

Conclusion

Many methods of C-banding have been developed during the last two decades, initially by Sumner (1972), which now becoming a standard method for C-band staining in plant and animal specimen. However, to optimize the method for specific species, researchers in their own laboratory must be doing a specific modification of the technique in order to result in good and readable banding. C-banding which so far have little been noted, was very useful to identify a whole arm translocation in small apes. Amid many very advanced techniques for chromosome analysis, C-banding still become a very important technique for cytogenetic analysis, because such method is very simple, cheap, and applicable for small laboratory in developing countries, which usually have only limited facilities.

Acknowledgement

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References


