



Genetic mechanism and property of a whole-arm translocation (WAT) between chromosomes 8 and 9 of agile gibbons (*Hylobates agilis*)

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Received 10 August 2002. Received in revised form and accepted for publication by Jennifer Graves 8 November 2002

Key words: C-band, 44-chromosome gibbons, *Hylobates agilis*, whole-arm translocation

Abstract

C-banding analysis with 47 gibbons of the subgenus *Hylobates* (*Hylobates*) (44-chromosome gibbons) uncovered that the gibbons had a characteristic complicated C-banding pattern. The C-band pattern also revealed that a whole-arm translocation (WAT) between chromosomes 8 and 9 existed only in the species *H. agilis* (agile gibbon). Comprehensive consideration allows postulation that the translocation seemed to be restricted to two subspecies: *H. agilis agilis* (mountain agile gibbon) and *H. agilis unko* (lowland agile gibbon), found in Sumatra and part of the Malay Peninsula. Moreover, combined intensive analyses of C-banding and chromosome painting provided strong evidence for a plausible evolutionary pathway of chromosome differentiation of chromosomes 8 and 9. The C-banded morph 8M^{1/c} seemed to be the primary type of chromosome 8 in the subgenus and to have altered into the three morphs through three pericentric inversions. The newest morph (8A^{M/ci}) produced by the third inversion exchanged the long arm for chromosome 9, and subsequently constructed the WAT morphs of 8/9A^{Mc/ct} and 9/8M^{1/ci}.

Introduction

Gibbons (family Hylobatidae) show a variable chromosome number ranging from 2n = 38 to 2n = 52, which provides a useful landmark to identify the subgenera of this family, *Hylobates* (*Hylobates*) (2n = 44), *H. (Bunopithecus)* (2n = 38), *H. (Nomascus)* (2n = 52), and *H. (Symphalangus)* (2n = 50) (Prouty *et al.* 1983, Marshall & Sugardjito 1986, Geissmann 1995). Most recently, however, it was proposed in a molecular analysis that these groups should be classified as four

genera (Roos & Geissmann 2001) because the molecular distances between the four gibbon subgenera were in the same range as those between *Homo* and *Pan*, or even higher. This view was also accepted by the Primate Taxonomy Workshop held 25 February–1 March 2000 (Mootnick, in press). Moreover, a subspecies *H. agilis albibarbis* (Bornean agile gibbon) was promoted to species rank, *H. albibarbis* (Bornean white-bearded gibbon) (Groves 2001). On the other hand, the Hylobatidae has a very differentiated karyotype from humans (Jauch *et al.* 1992, Koehler *et al.*

1995, Nie *et al.* 2001). Interestingly, Old World monkeys are closer to great apes and humans at the chromosomal level than are the Hylobatidae (Weinberg *et al.* 1992), although the phylogenetic relationship is rather far. The chromosome evolution and phylogeny of the *Hylobatidae* still remains elusive.

In the subgenus *H. (Hylobates)*, interspecific pericentric inversion polymorphisms have been reported using the G-banding technique (e.g. Tantravahi *et al.* 1975, Stanyon *et al.* 1987). More recently, it was found that the inversion polymorphism was accompanied by a reciprocal translocation (whole-arm translocation) between chromosomes 8 and 9 (vanTuinen *et al.* 1999). This startling fact was found by observing the synaptonemal complex (SC) formation in the meiotic pairing. These chromosomal traits may be manifestations of chromosomal differentiation or of speciation in the subgenus. We have also found that the gibbons unexpectedly have the complicated C-banding patterns, although the previous data demonstrated a simple centromeric-only pattern (vanTuinen & Ledbetter 1983). The complicated C-band pattern also embossed the complex polymorphism of chromosomes 8 and 9. Although this polymorphism cannot be consistently defined only from complex inversions, the findings of vanTuinen *et al.* (1999) definitely allowed us to clarify obscure manifestations of the compound polymorphism. In this paper, we report novel C-banded karyotypes in gibbons and discuss a further plausible pathway of rearrangements of chromosomes 8 and 9 using C-banding and painting analyses, and the origin of the whole-arm translocation (WAT). Moreover, we argue that genetic or geographical isolation might have occurred between Bornean agile gibbons and the other two subspecies (lowland agile gibbons (*Hylobates agilis unko*) and mountain agile gibbons (*H. a. agilis*)).

Materials and methods

Samples

Species identification was made from the pelage and facial traits (Marshall & Sugardjito 1986, Geissman 1995, Mootnick 2002), which in some

species was only tentative. The identification of some gibbon species and subspecies based exclusively on morphological features alone may not always be practical, even for the specialist (Geissmann 1995). In some cases, vocal analysis will resolve the uncertainty when accurate species identification is required (Marshall & Sugardjito 1986). Except for one male #45, we were unable to record the vocalization of the gibbons from which we obtained blood samples. We identified 47 individuals of the following four species: *Hylobates agilis*, *H. muelleri* (Mueller's gibbon), *H. moloch* (Java gibbon), and *H. lar* (white-handed gibbon). Species or subspecies with an asterisk (*) in Table 1 were identified by one of us (ARM) using photographs. Blood samples were collected from captive gibbons that were in the possession of pet owners, three zoos, and one institute (Table 1). Most of the pets were captured from their native habitat close to the individual house. It should also be noted that Pangkalang Buun, Kalimantan, is an area far from a hybrid zone for *H. agilis albibarbis* and *H. muelleri* and is an area where *H. a. albibarbis* is native. On the other hand, for this study, the exact birth location of wild-born gibbons or the parentage of gibbons born in zoological institutions was generally unknown. Captive breeding is carried out in many zoological institutions for saving species, but, ironically, it can also create a problem if hybridization occurs.

Whole blood (less than 3 ml/kg) was collected with a heparinized syringe from gibbons anaesthetized with ketamine hydrochloride (less than 10 mg/kg by an intramuscular injection).

Chromosome preparation

One milliliter of whole blood was cultured with 9 ml RPMI 1640 medium (Nipro, Japan) containing the following agents: 20% FCS (Gibco BRL, USA), a three-mitogen mixture (10 µg/ml phytohaemagglutinin (Murex Biotech, UK), 3 µg/ml concanavalin A (Sigma, USA), and 3 µg/ml lipopolysaccharide (Sigma, USA)), 50 µg/ml streptomycin, and 50 U penicillin. The culture was made in a 37°C CO₂ incubator for 70 h. After treating the culture with 50 µg/ml colcemid (Gibco BRL) for 30 min, lymphocytes were harvested by

Table 1. C-band polymorphism of chromosomes 8 and 9 in 44-chromosome gibbons.

Sample No.	Species	Sex	Sampling site	Situation of animal	#8 morphs	#9 morphs	Sampling date
1	*HAGAL	F	PBK	pet	A ^{Mci} A ^{Mci}	M ^h M ^h	19/09/96
2	*HAGAL	F	PBK	pet	A ^{Mci} M ^{tc}	M ^h M ^h	19/09/96
3	*HAGAL	M	PBK	pet	A ^{Mci} M ^{tc}	M ^h M ^h	19/09/96
4	*HAGAL	F	PBK	pet	A ^{Mci} M ^{cti}	M ^h M ^h	19/09/96
5	*HAGAL	M	PBK	pet	A ^{Mci} A ^{Mci}	M ^h M ^h	19/09/96
6	*HAGAL	M	PBK	pet	A ^{Mci} M ^{cti}	M ^h M ^h	19/09/96
7	*HAGAL	F	PBK	pet	A ^{Mci} M ^{cti}	M ^h M ^h	19/09/96
8	*HAGAL	F	PBK	pet	A ^{Mci} A ^{Mci}	M ^h M ^h	19/09/96
9	*HAGAL	M	PBK	pet	A ^{Mci} M ^{tc}	M ^h M ^h	19/09/96
10	*HAGAL	M	PBK	pet	A ^{Mci} A ^{Mci}	M ^h M ^h	19/09/96
11	*HAGAL	F	WAK	pet	A ^{Mci} A ^{Mci}	M ^h M ^h	30/09/96
12	*HMUMU	F	SAK	pet	A ^{Mci} M ^{tc}	M ^h M ^h	28/09/96
13	*HMUMU	F	BAK	pet	A ^{Mci} A ^{Mci}	M ^h M ^h	29/09/96
14	*HMUMU	M	BAK	pet	A ^{Mci} M ^{cti}	M ^h M ^h	29/09/96
15	*HMUMU	F	BAK	pet	A ^{Mci} M ^{cti}	M ^h M ^h	29/09/96
16	*HMUMU	F	BAK	pet	A ^{Mci} A ^{Mci}	M ^h M ^h	29/09/96
17	*HMUMU	M	BAK	pet	A ^{Mci} M ^{cti}	M ^h M ^h	29/09/96
18	*HMUMU	M	BZK	captive	A ^{Mci} M ^{tc}	M ^h M ^h	02/10/96
19	*HMUMU	F	BZK	captive	M ^{cti} M ^{cti}	M ^h M ^h	02/10/96
20	*HMUMU	F	BZK	captive	M ^{tc} M ^{tc}	M ^h M ^h	02/10/96
21	*HMUMU	M	BZK	captive	M ^{cti} M ^{cti}	M ^h M ^h	02/10/96
22	*HAGAL	F	BZK	captive	A ^{Mci} A ^{Mci}	M ^h M ^h	02/10/96
23	*HAGUN	M	RZJ	captive	A ^{Melet} A ^{Melet}	M ^{ici} M ^{ici}	22/09/96
24	*HAGAG	F	RZJ	captive	M ^{cti} A ^{Melet}	M ^h M ^{ici}	22/09/96
25	*HAGUN	M	RZJ	captive	M ^{tc} A ^{Melet}	M ^h M ^{ici}	22/09/96
26	*HAGAL	M	RZJ	captive	A ^{Melet} A ^{Melet}	M ^{ici} M ^{ici}	22/09/96
27	*HAGUN	M	RZJ	captive	A ^{Melet} A ^{Melet}	M ^{ici} M ^{ici}	22/09/96
28	*HAGUN	F	RZJ	captive	A ^{Melet} A ^{Melet}	M ^{ici} M ^{ici}	22/09/96
29	*HAGUN	M	RZJ	captive	M ^{cti} M ^{cti}	M ^h M ^h	22/09/96
30	*HMUFU	M	RZJ	captive	M ^{tc} M ^{tc}	M ^h M ^h	22/09/96
31	*HMUFU	F	RZJ	captive	M ^{tc} M ^{tc}	M ^h M ^h	22/09/96
32	*HMO	F	RZJ	captive	A ^{Mci} M ^{tc}	M ^h M ^h	22/09/96
33	*HMO	M	RZJ	captive	M ^{tc} M ^{tc}	M ^h M ^h	22/09/96
34	HMO	M	RZJ	captive	A ^{Mci} M ^{tc}	M ^h M ^h	22/09/96
35	*HMUFU	M	RZJ	captive	A ^{Mci} M ^{tc}	M ^h M ^h	22/09/96
36	*HMUFU or HAG or hybrid	F	RZJ	captive	A ^{Mci} M ^{cti}	M ^h M ^h	22/09/96
37	HAG	F	JMC	captive	A ^{Mci} M ^{cti}	M ^h M ^h	09/03/94
38	*HAGUN	F	JMC	captive	M ^{tc} A ^{Melet}	M ^h M ^{ici}	09/03/94
39	*HAGUN	F	JMC	captive	A ^{Melet} A ^{Melet}	M ^{ici} M ^{ici}	09/03/94
40	*HLA	M	JMC	captive	M ^{tc} M ^{tc}	M ^h M ^h	09/03/94
41	*HMUFU	F	JMC	captive	A ^{Mci} M ^{tc}	M ^h M ^h	09/03/94
42	HLA	M	PRI	captive	M ^{tc} M ^{tc}	M ^h M ^h	21/04/94
43	*HLA	F	PRI	captive	M ^{tc} M ^{tc}	M ^h M ^h	21/04/94
44	*HAGUN	F	PRI	captive	M ^{cti} M ^{cti}	M ^h M ^h	25/05/94
45	* ⁺ HAGAL	M	PRI	captive	A ^{Melet} A ^{Melet}	M ^{ici} M ^{ici}	25/05/94
46	44/45	M	PRI	captive	M ^{cti} A ^{Melet}	M ^h M ^{ici}	30/09/00
47	44/45	M	PRI	captive	M ^{cti} A ^{Melet}	M ^h M ^{ici}	30/09/00

HAG, *H. agilis*. HAGAL, *H. agilis albibarbis*. HAGUN, *H. agilis unko*. HAGAG, *H. agilis agilis*. HMU, *H. muelleri*. HMUFU, *H. muelleri funereus*. HMO, *H. moloch*. HLA, *H. lar.* -*, individual identified by photographs by Alan Mootnick. *⁺, individual identified by photographs and vocalization by Alan Mootnick. F, female. M, male. PBK, Pangkalan Buun, Kalimantan. SAK, Samarinda, Kalimantan. WAK, Wanawisata, Kalimantan. BAK, Balikpapan, Kalimantan. BZK, Banjarmasin Zoo, Kalimantan. RZJ, Ragunan Zoo, Jakarta. JMC, Japan Monkey Center, Japan. PRI, Primate Research Institute, Kyoto University, Japan. Pet was a wild-born gibbon that was raised by a private individual. Captive was a gibbon that was raised in the zoo or institution with unknown birth origin. #8, chromosome 8. #9, chromosome 9. Sampling date: day/month/year. Solid line indicates difference of area and/or raising condition.

centrifugation and fixed with ethanol and acetic acid (3:1). Details of the slide preparation were previously described (Hirai *et al.* 1998, 1999).

C-banding

C-banding procedures were done using a technique slightly modified from the method of Sumner (1972). Briefly, after treatment with 0.2 N HCl, the chromosome preparation was treated with a 5% BaOH solution at 55°C for 5 min and 2 × SSC at 55°C for 20 min, and finally, stained for 2 h with a 4% Giemsa solution in pH 6.8 Sörensen's phosphate buffer. During the intervals between each treatment, the slide preparation was sufficiently washed with running tap water. Identification of C-banded chromosomes was made with post-G-band C-banding (G-C banding). However, as the G-C banding process often makes original C-bands dull-positive, a direct C-banding was indispensable for identifying the C-banded karyotype. To confirm the identification, C-banded chromosomes were defined by collating the number of G-banding patterns (vanTuinen & Ledbetter 1983) with the number of chromosomes through G-C banding. Direct C-banded karyotypes obtained from 47 individuals were examined individually through a comparing analysis of each chromosome using printed pictures of 2–5 good chromosome spreads in each gibbon.

Terminology for C-banded chromosomes

Since C-banded karyotyping of the 44-chromosome gibbon demonstrated a complicated pattern, an appropriate nomenclature was required for defining the C-banded karyotype of the species. We adopted Imai's TAM system (Imai 1991, Imai *et al.* 2001) to classify C-banded chromosomes. This system was very useful for identifying chromosomes with C-band variations. According to the terminology, the basic nomenclature of C-banded karyotypes, A, and A^M, M^A, and M, is defined such that A and A^M have the heterochromatic short and euchromatic long arms, and M^A and M have two euchromatic arms (See also Figure 1a). This is based on the basic categories and the 'cit' nomenclature system for describing

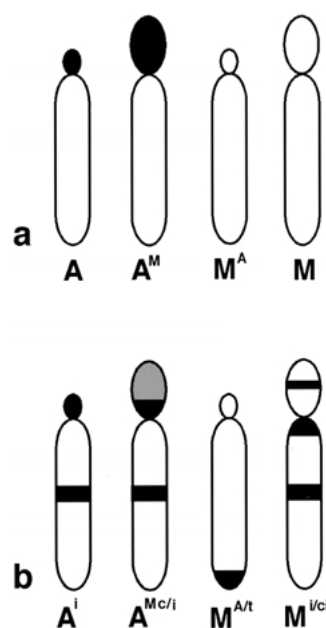


Figure 1. Schematic explanation for nomenclature of C-banded karyotypes. See text for details.

the C-band pattern (Imai 1991), which allows c, i and t to be the C-bands located at the pericentromeric, interstitial, and terminal regions of the chromosome arms, respectively. Each gibbon chromosome was classified in more detail using specific bands. A slash (/) between the superscript letters indicates the band location. The left side is in the short arm and the right in the long arm (Hirai *et al.* 2002). For example, Aⁱ indicates that the A chromosome has an interstitial C-band in the long arm; the A^{M^c/i}, A^M chromosome has a pericentromeric C-band in the short arm and an interstitial C-band in the long arm; the M^{A^t}, M^A chromosome has a terminal C-band in the long arm; and the M^{i/ci}, M chromosome has an interstitial C-band in the short arm and a pericentromeric and an interstitial C-band in the long arm (See also Figure 1b). Generally, the A^M chromosome does not require the slash because the short arm of these chromosomes is totally heterochromatic. However, gibbons need designation of pericentromeric C-band in A^M, since the heterochromatic short arm of the A^M chromosome has two differentiated types in the C-band, dark and light.

Chromosome painting analysis

In order to ensure the chromosome variations observed with C-banding techniques, the painting analyses were conducted using painting probes of human chromosome (HSAC). According to the analysis of genomic rearrangements in gibbons using all human painting probes (Jauch *et al.* 1992), standard chromosome 8 of *Hylobates lar* is composed of fragments of HSACs 16, 5, 22, 17 and 9 from the top of the short arm to the bottom of the long arm. The three painting probes of HSACs 5, 22, and 17 were finally selected as probes to examine rearrangements in chromosomes 8 and 9 of 44-chromosome gibbons, since these probes were thought to be convenient for detecting inversion sites including the centromeric region and translocation between both the chromosomes.

Painting analyses were made by a fluorescent *in-situ* hybridization (FISH) technique with three probes, Rhodamine-labelled HSAC 5 (Appligene-Oncor, CP5605-RW), FITC-labelled HSAC 17 (Appligene-Oncor, CP5617-GW), and Dig-labelled HSAC 22 (Roche, Germany). Briefly, denaturation of chromosomal DNA was made with pH 12.5 $2 \times$ SSC solution for 4.5 min and/or 74°C 70% formamide in $2 \times$ SSC for 3 min, followed by dehydration with 70% and 99.5% ethanol chilled in ice. The probes were applied to a denatured slide with a mixture of 10 μ l Dig-HSAC 22 probe for the single-FISH, and a mixture of 5 μ l each direct labelled probe (5 and 17) for the double-FISH. After denaturation at 74°C for 10 min and pre-annealing at 37°C for 30 min, the probe mixture was set onto a slide. After 16 h hybridization and post-hybridization washing, the 22 probe signals were detected using a mixture of anti-Dig-antibody-FITC (Roche) in 5% non-fat milk BN (0.5 mol/L sodium bicarbonate and 0.1% NP-40) buffer and counterstain with anti-fade solution containing 100 ng DAPI and PI (Hirai *et al.* 1999). Dual FISH with HSAC 5 and 17 probes was directly observed after counterstaining with anti-fade solution containing 200 ng DAPI. The results were observed using a Zeiss Axiophoto fluorescence microscope and saved into a computer (Macintosh, 8500/120) with a cooled charge-coupled device camera system (PXL, Photometrics, USA). Chromosome classification was performed using post-FISH DAPI- and C-bands.

The post-FISH C-banding was made as previously described (Hirai 2001).

Results*C-banding pattern and variations of chromosomes 8 and 9*

Figure 2 shows a representative C-banded spread of a female *H. agilis unko*. As shown in the plate, all chromosomes showed a specific C-banded pattern. Some C-banded chromosomes (with number) were identified in terms of matching to the G-banded karyotype (vanTuinen & Ledbetter 1983), which was done using G-C-banding, but others are still inconsistent with the previous G-banding. For example, chromosome 1 was $M^{c/}$; chromosome 3, $M^{/c}$; chromosome 7, M^{ci} ; chromosome 11, $M^{t/ct}$; chromosome 12, $M^{t/}$; chromosome 16, $M^{t/ct}$; and chromosome 21, A^M . On the other hand, chromosomes 8 and 9 showed a mixture of both elements (Figure 2, 8/9 and 9/8) due to a whole-arm translocation (WAT) which will be described in more detail later. We detected a total of seven types in both chromosomes 8 and 9. The C-banded karyotypes of the variants were respectively designated as $A^{M/i}$, $A^{Mc/ct}$, $M^{t/c}$, $M^{ct/i}$, $M^{i/ci}$ and $M^{i/t}$ (Figures 3a,b). Since the $A^{M/i}$ was divided into two subtypes, $A^{M/ci}$ and $A^{Mc/i}$ due to the location of centromere (Figure 3 inset), we observed a total of seven different morphs in chromosomes 8 and 9. Sequential G-C-banding and FISH-C-banding allowed us to identify these chromosomes as chromosomes 8 and 9 defined by vanTuinen & Ledbetter (1983) (Figures 3c, d & 5).

Finally, painting analyses with probes of HSACs 5, 17 and 22 precisely clarified the identification and elucidated the mechanism of such variations. The technique effectively supported elucidating a plausible pathway of chromosome differentiation through signal locations and post-FISH DAPI- and C-bands. Figure 4 demonstrates hybridization patterns detected with two HSAC probes (5, red and 17, green) in two different variants (a and b). The hybridization was similar to previous data (Jauch *et al.* 1992). Chromosome 9, which was classified by vanTuinen & Ledbetter (1983), has a large block of HSAC 17 in the short arm. According to comparison with other previous

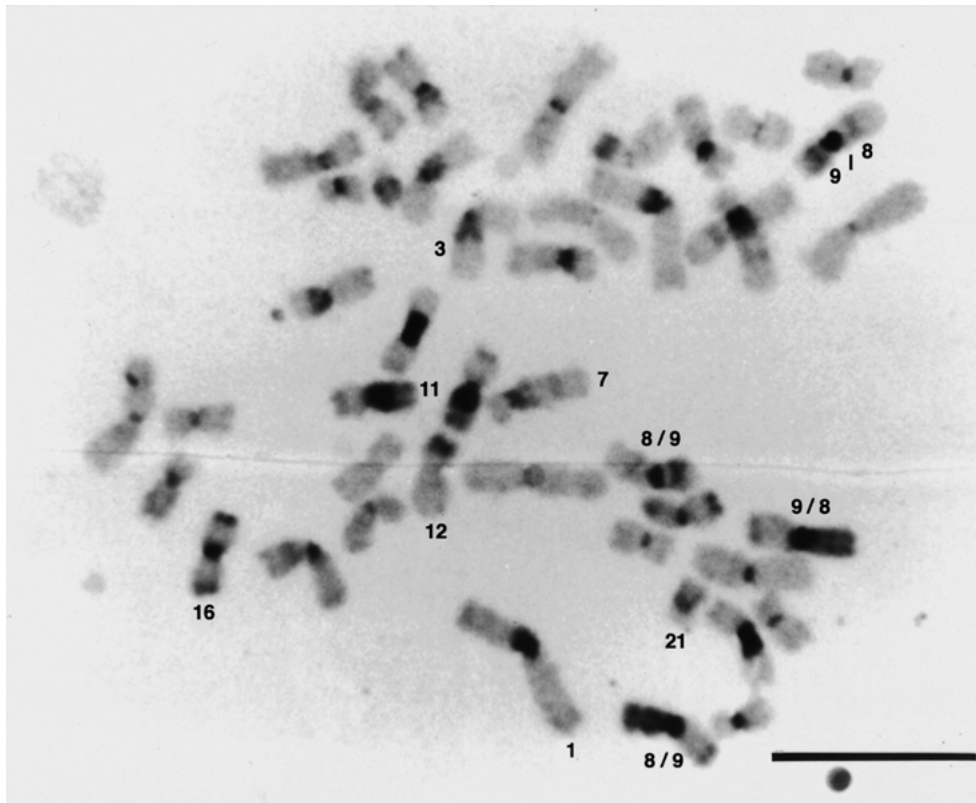


Figure 2. Direct C-banded chromosome spread of a female *H. agilis unko*. Numbers indicate each chromosome. Scale bar = 10 μ m.

data, this chromosome has been classified as chromosome 13 by Jauch *et al.* (1992) and as chromosome 12 by Stanyon & Chiarelli (1983).

Figure 5 comprehensively shows the results of the painting analyses, DAPI- and C-bands in each chromosomal variant and mechanisms solved from location of each chromosome element. Painting analyses uncovered that the $8M^{t/c}$ has the simplest composition of the three HSAC probes. HSAC 22 probe (red arrowhead), being located at the interval site between HSACs 5 and 17, detected a medium-sized pericentric inversion (m.pe.in.) on chromosome 8, which transferred a small amount of HSAC 22 to the short arm (two red arrowheads), resulting in the $8M^{ct/i}$. HSACs 5 and 17 disclosed a larger pericentric inversion (l.pe.in.) on chromosome 8 which transferred most HSAC 22 to the short arm and left a small amount in the long arm (two red arrowheads), and most HSAC 5 (pinkish signal) to the long arm and left a small amount in the short arm, resulting in the $8A^{Mc/i}$ and the $8A^{M/ci}$. The $8A^{M/ci}$ might be induced by a

smaller pericentric inversion (s.pe.in.) in pericentric heterochromatin from the $8A^{Mc/i}$ (inset) because the l.pe.in. inevitably reconstructs the $8A^{Mc/i}$ from the $8M^{t/c}$. Jauch *et al.* (1992) possibly used the $8M^{ct/i}$ for painting analysis of gibbon chromosomes, since the data indicate the same pattern in our painting study.

Chromosome 9 showed the large block of HSAC 17 (green signal in the first left chromosome) on the short arm, being a good landmark to identify the chromosome ($9M^{t/i}$) together with C-band and other bands (yellow and green arrowheads). On the other hand, the tandem location of HSACs 5 (pink) and 17 (green) in the long arm of the $8A^{M/ci}$ precisely discovered that the long arm transferred to chromosome 9 with the characteristic short arm. Additionally, other traits (arrow heads) of both chromosomes also indicated the mixture condition of chromosomes 8 and 9. The $8/9A^{Mc/ct}$ and $9/8M^{i/ci}$ might have been reconstructed by WAT between chromosomes 8 and 9. A mechanism for forming the WAT will be shown in

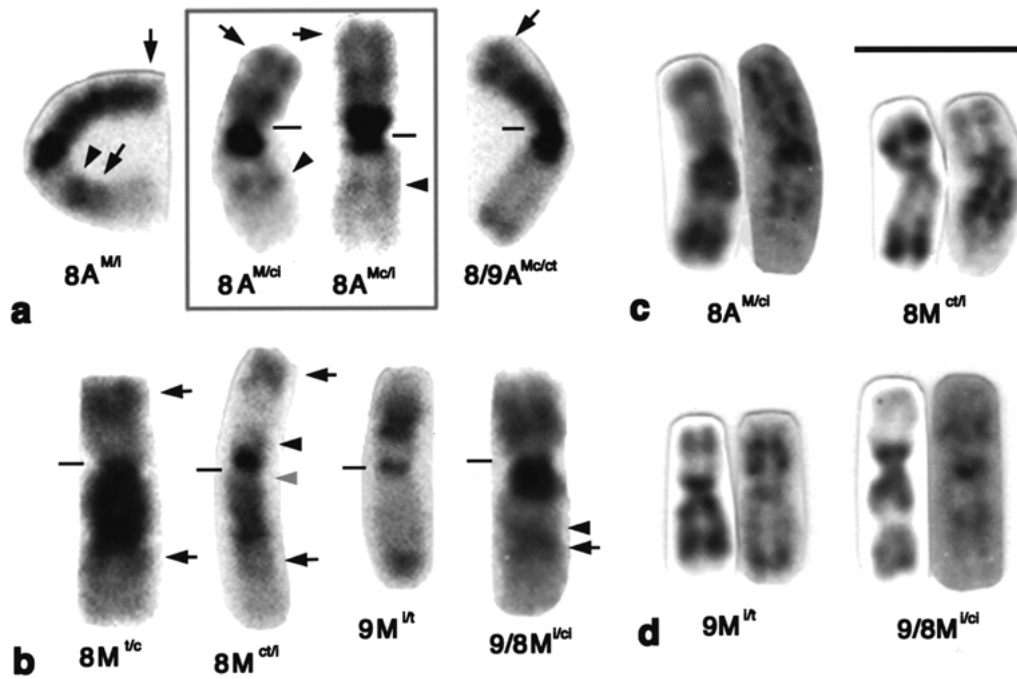


Figure 3. C-banded variants of chromosomes 8 and 9 of the subgenus *H.* (*Hylobates*). (a) A-chromosome. (b) M-chromosome. (c) G-C-banding for chromosome 8 (left side is G-band and right C-band). (d) G-C-banding for chromosome 9 (left side is G-band and right C-band). Small solid bar indicates centromere region; Arrowhead (dark C-band) and arrow (light C-band) show two differentially stained C-bands, respectively; grey arrowhead points out euchromatic gap. Scale bar = 10 μ m. See text for details.

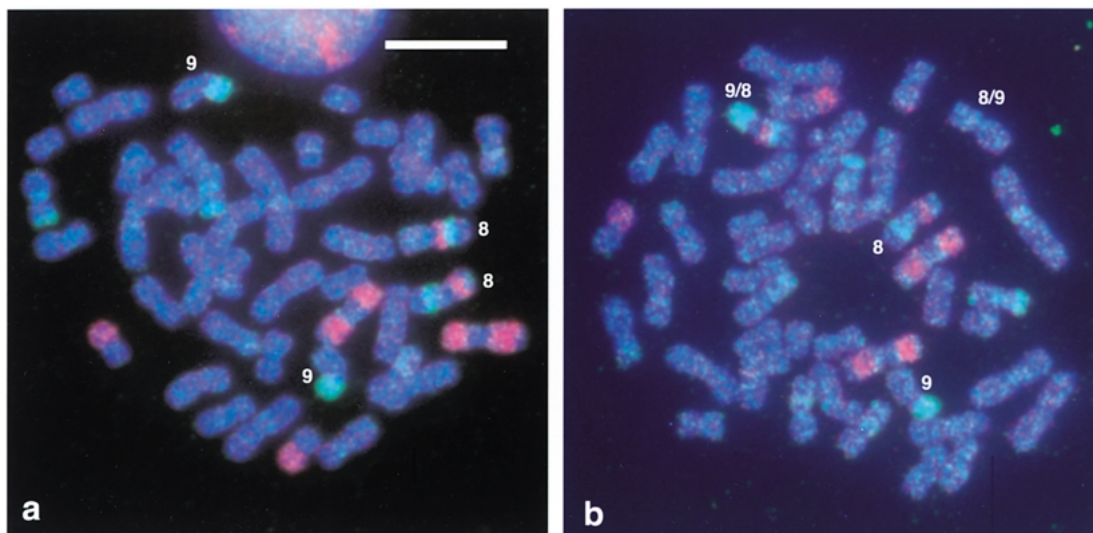


Figure 4. FISH images analysed with human chromosome (HSAC) painting probes 5 (pink) and 17 (green) to two gibbons heterozygous for larger inversion (a) and for a whole-arm translocation (WAT) (b). Numbers indicate each chromosome.

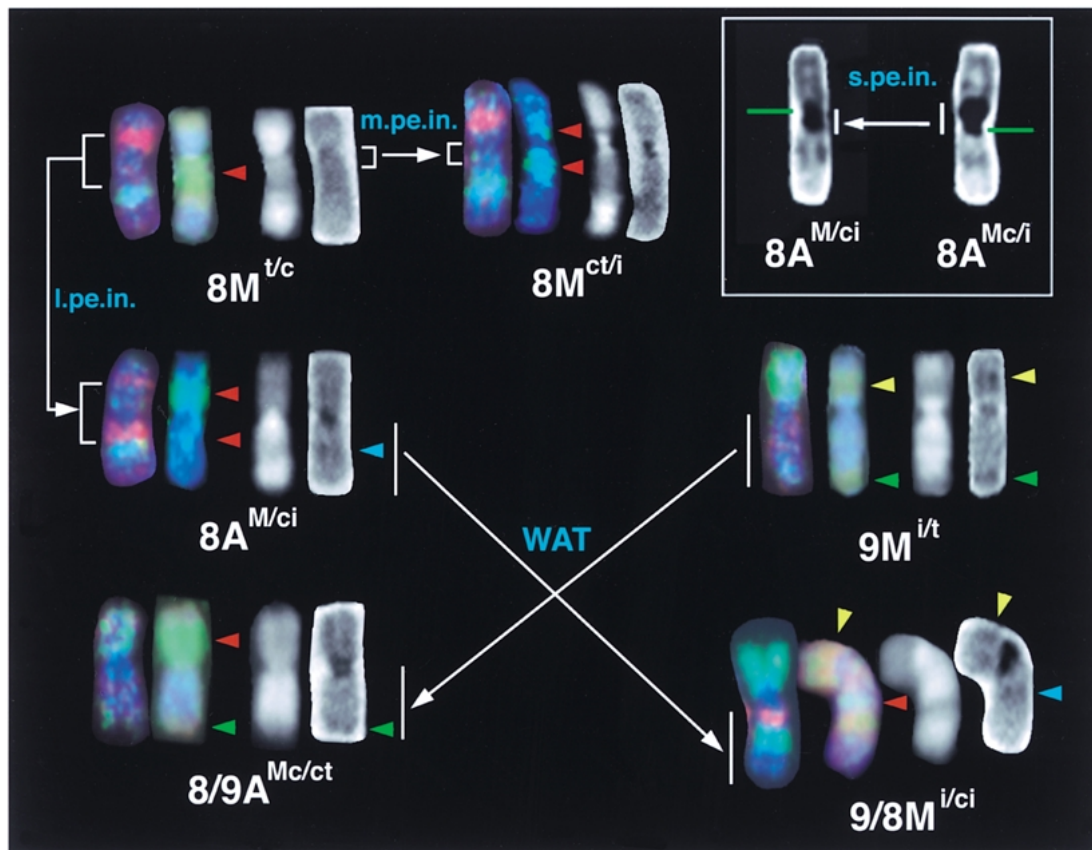


Figure 5. Comprehensive analysis of differentiation of chromosomes 8 and 9 in agile gibbons by dual FISH with HSAC 5 and 17 probes, single FISH with HSAC 22 probe, post-FISH DAPI- and C-banding, from left to right. 8 and 9 indicate the chromosome number; l.pe.in., large pericentric inversion; m.pe.in., medium-sized pericentric inversion; s.pe.in., small pericentric inversion; WAT, whole-arm translocation. Red arrowhead indicates signal HSAC 22; blue arrowhead points out an interstitial C-band in the long arm of chromosome 8; yellow (short arm) and green (long arm) arrowheads show natural bands stained with propidium iodide (PI) after the FISH procedure, corresponding with C-bands in chromosome 9. Note that FISH images of $A^{M/ci}$ and $M^{ct/i}$ hybridized with HSAC 22 were printed out by two colours of FITC and DAPI but others by three colors of FITC, DAPI and PI. Inset indicates difference of centromere location (green bar) of two morphs. White arrow shows direction of chromosome alteration. See text for details.

discussion. These characteristics are good diagnostic markers for identifying chromosomal rearrangements between chromosomes 8 and 9.

Genetic structure of the variants

The comprehensive examination of C-band polymorphisms on the 47 gibbons demonstrated that there appears to be a finite set of chromosomal variants occurring in chromosomes 8 and 9 (Table 1). Note that the $A^{Mc/ct}$ morph is always accompanied by the $M^{i/ci}$ morph (Table 1, shaded). Neither of the two morphs appeared to

combine with any other morph. This might have been induced by the deletion and duplication of chromosomal elements (genome imbalance) due to combining of non-translocated and translocated elements. That is, gametes with such an imbalanced genome are naturally selected by lethality. The WAT between chromosomes 8 and 9 was observed only in *H. agilis*. Twenty-four out of 45 (excluding offspring #46 and #47) gibbons examined were identified as *H. agilis*. Of those 24 gibbons, 14 were *H. agilis albibarbis*; 8 were *H. agilis unko*; 1 was *H. agilis agilis*; and 1 was unclear as to the subspecies. Only 2 (14%) of *H. a.*

albibarbis (#26 and #45) had the WAT. Note that the two *H. a. albibarbis* with the WAT were housed in zoological institutions, and the remaining 11 *H. a. albibarbis*, which were pets from Kalimantan, did not have the WAT. In contrast, 78% (7/9) of the *H. agilis unko* and *H. a. agilis* showed the WAT as homozygote (4) or heterozygote (3) (Table 1). The *H. lar*, *H. muelleri*, and *H. moloch* have never shown the WAT.

Discussion

Plausible pathway of the variations

A previous cytogenetic analysis (vanTuinen & Ledbetter 1983) showed that C-banding of the subgenus *H. (Hylobates)* was a simple centromeric pattern in all chromosomes. However, as observed in the present study, all the species in the subgenus seem to latently have the complicated C-banding patterns (Figure 1). Such sensitive C-bands were often suppressed through G-banding and/or FISH treatment. This is probably a reason why the previous paper described only a simple centromeric C-band pattern. A similar complicated C-banding pattern was also described in the 38-chromosome subgenus, *Hylobates (Bunopithecus) hoolock* (Ruiqing *et al.* 1987).

Using the principle of parsimony for chromosome rearrangements, a plausible pathway can be constructed as follows (Figure 6). The painting analyses used here postulated that $8M^{t/c}$ is probably the original karyotype of the compound variations occurring in chromosome 8 of gibbons, since $8M^{t/c}$ has the simplest signal found only in the long arm, and is not related to the WAT (Figure 5). If the larger and medium-sized pericentric inversion occurred on the $8M^{t/c}$, then $8A^{Mc/i}$ and $8M^{ct/i}$ could be derived, respectively (Figure 5). These estimations were discovered through transpositions of the signals into the long arm and the short arm, respectively. Both of the small signals of HSAC 22 correspond with the small C-bands in the two arms; thus the two marks should be footprints of the inversions that occurred earlier (Figures 3b & 5). The $8A^{M/ci}$ can be derived by allocation of centromere through a small pericentric inversion in pericentric

heterochromatin of the $8A^{Mc/i}$ that was described by Stanyon *et al.* (1987). This can be inferred by the transposition of centromere (Figures 3 & 5 inset, black or green bar), although the shift of centromere within pericentromeric C-band also can be postulated. Additionally, a WAT between chromosomes $8A^{M/ci}$ and $9M^{i/t}$ induced $8/9A^{Mc/ct}$ (8 short arm + 9 long arm) and $9/8M^{Mi/ci}$ (9 short arm + 8 long arm) (Figures 5 & 6). Existence of the WAT was recognized with interstitial paint signals on the long arm of $8A^{M/ci}$ and post-FISH DAPI- and C-bands. The feature of pericentric C-bands (c) in the long arm of $9/8M^{i/ci}$ and $8/9A^{Mc/ct}$ (Figure 3) also allowed us to perceive that the WAT occurred between $8A^{M/ci}$ (not $8A^{Mc/i}$) and $9M^{i/t}$ (Figure 5). Moreover, it can be defined from C-band traits that the WAT occurred between two breakage points of a region in the pericentric heterochromatic block of the $8A^{M/ci}$ long arm and a centromeric region of the $9M^{i/t}$ long arm (Figure 6, white arrowheads).

In addition, it is also generally postulated that the original morph ($8M^{t/c}$) of the compound variations might have evolved from one ancestral morph ($A^{Mc/c}$) because the morph indicates one step previous stage of the $8M^{t/c}$, estimated by the theory of AM inversion cycle (Imai 1991). Though observations with out-group animals might be required in order to find the postulated original morph, a pericentric inversion occurring in the original morph could have created the $8M^{t/c}$ (Figure 6). As illustrated in Figure 6, such speculation is supported by two differentially stained interstitial C-bands of the short and long arms of chromosome 8, which consist of two different heterochromatic bands (dark and light). The dark C-band appears to be included in a pink signal hybridized with HSAC 5 probe and the light band appears to be included in a green signal hybridized with HSAC 17 probe (Figure 5). Thus, these bands may have different DNA compositions from each other. Moreover, A^{Mi} and $M^{t/c}$ of a direct C-banding showed two differentially stained C-bands, dark and light (Figures 3a, 3b, arrowhead and arrow). These two different C-bands were both very weak bands in the long arm, and the stain condition was not as stable as the pericentromeric C-band. Such bands may have the threshold copy numbers of long-range repeats that can be detected as C-bands. Since the differential staining beha-

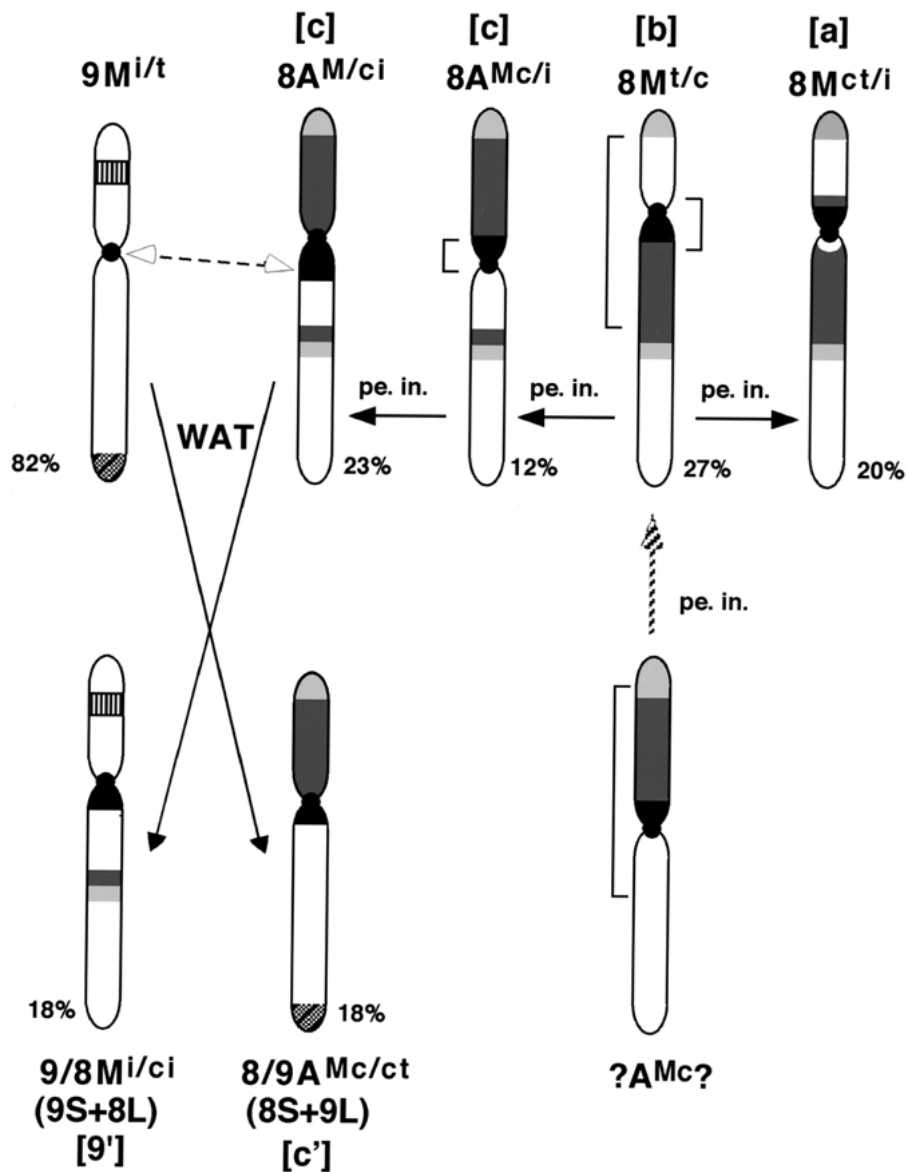


Figure 6. A plausible pathway of chromosome rearrangements in 44-chromosome gibbons. White region, euchromatin; black region, centromeric or pericentromeric C-band; two different shadow regions indicate two differentially stained C-bands (dark and light). pe. in., pericentric inversion; WAT, whole-arm translocation; bracket on side of chromosome shows breakage point of inversion; white arrowheads with broken line show breakage points of the WAT; percentages indicate (%) occurrence frequency in all gibbons examined here. Letters of the alphabet in large parentheses shows variant morph used in previous studies; 8 and 9 is each chromosome number; S and L, short and long arm; question mark indicates a theoretically estimated ancestral morph. See text for details.

viour depends on the copy number, then the threshold appears to be between 105 and 175 copies with a repeat size of roughly 100 kbp per haploid (Kunze *et al.* 1996). Since the short arms of $8A^{M/ci}$ and $8A^{Mc/i}$ are composed of two

differentially stained bands, this also supports the existence of the postulated ancestral chromosome, $A^{Mc/}$. C-banded karyotypes $8M^{ct/i}$, $8M^{t/c}$, $8A^{Mc/i}$, $8A^{M/ci}$ and $8/9A^{Mc/ct}$ correspond with chromosome 8 morphs described previously as a, b, c, c,

Table 2. Occurrence frequency of WAT between chromosomes 8 and 9 found in each subspecies of agile gibbons.

Subspecies	WAT frequency						Total frequency of WAT (%)
	This study			vanTuinen <i>et al.</i> (1999)			
	+/+	+/-	-/-	+/+	+/-	-/-	
<i>H. a. agilis</i>	0 ^a	1	0	0	4	7	19 ^c /24 ^b (79)
<i>H. a. unko</i>	2	2	4	0	3	1	15/24 (63)
<i>H. a. albibalbis</i>	12	0	2	2	1	0	5/34 (15)

WAT: whole-arm translocation between chromosomes 8 and 9. +/+, homozygote of normal. +/-, heterozygote of normal and WAT. -/-, homozygote of WAT. ^a individual number. ^b total chromosome number of chromosome 8. ^c total number of chromosome 8 with WAT.

and *c'* (Stanyon *et al.* 1987, vanTuinen *et al.* 1999), respectively (Figure 6).

Origin of the WAT

A combined analysis of chromosome variations found in a previous study by vanTuinen *et al.* (1999) and in the present study provided us with important information related to the origin of the WAT between chromosomes 8 and 9. Both studies suggest that the translocation appeared to originate in an ancestral population of *H. a. agilis* and *H. agilis unko* because the WAT seems to be restricted to the two subspecies. According to data from the two studies, 79% and 63% haploid genome sets of *H. a. agilis* and *H. agilis unko*, respectively, have the WAT, but *H. a. albibalbis* shows it in only 15% (Table 2). The frequencies of the two former and the latter could be significantly different from each other. Additionally, the three variants of *H. a. albibalbis* were of unknown parentage in zoological institutions. Such captive animals have often created problems for species management, as discussed below. Moreover, 11 pets of *H. a. albibalbis* that were randomly sampled in Kalimantan (Table 1) and the two wild-born *H. a. albibalbis* from a previous study (vanTuinen *et al.* 1999) did not have the WAT. Since the former pets appear to be wild-born around the area, then the natural populations of *H. a. albibalbis* may not have the WAT. Of course, a final conclusion of the matter has to be deferred until more intensive analyses using wild-born animals of Sumatran and Bornean (Kalimantan) natural populations.

If the above hypothesis is true, the mutation is going to be fixed only in the two subspecies due to the geographical isolation and/or genetic mechanism. That is, the WAT is useful as a landmark to distinguish *H. a. agilis* and *H. agilis unko* from *H. a. albibalbis*. A recent monograph elevated the *Hylobates (H.) agilis albibalbis* to a species level *H. albibalbis* (Groves 2001), which would be supported by our recent findings. *H. a. agilis* are found exclusively in the west Sumatra highlands; *H. agilis unko*, in the lowlands east and west Sumatra and an area between the Mudo and Perak rivers of the Malay Peninsula lowlands (Mootnick, in press). *H. agilis albibalbis* is found in Western and Central Kalimantan between the Kapuas and Barito rivers (Marshall & Sugardjito 1986, Geissman 1995). Why is the mutation restricted to two of the three subspecies of *H. agilis*? During the last glacial period, the Sunda Shelf has frequently changed the sea level, ranging from 50 m to 120 m lower than at present (Voris 2000). In addition, the North and East Sunda Rivers that existed between Belitung Island and Borneo and between Java and Borneo, respectively, bisected the Sunda Shelf, which was exposed during the driest periods (Tjia 1980) (see Figure 7). Although it is not clear when the three subspecies of *H. agilis* were isolated, both the lower sea level and the river systems might have served as barriers for geographical isolation on the Sunda Shelf. Under this scenario, it might also be possible that *H. agilis* migrated to Borneo and hybridized with *H. muelleri*, and that their descendants (*H. a. albibalbis*) evolved with vocalizations similar to *H. agilis*, but with chromosomes and pelage more similar to *H. muelleri*.

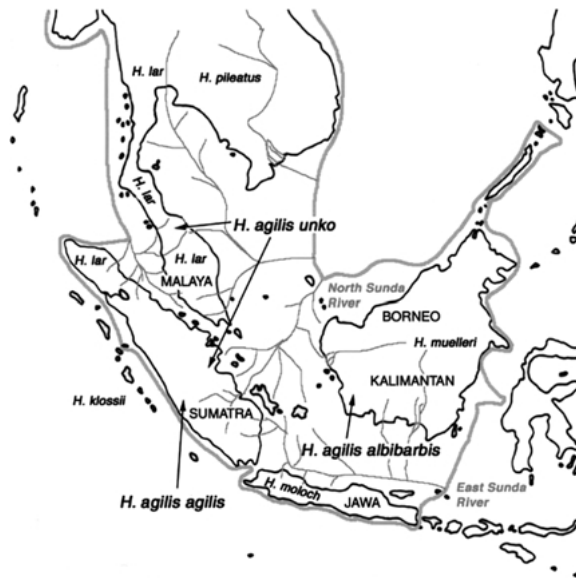


Figure 7. Map of the Sunda Shelf showing the sea level and the river systems at the glacial epoch and distribution of the species and subspecies of the subgenus *H.* (*Hylobates*). Wide grey lines show the depth contour line of 120 m below the present level (solid black line). Narrow grey lines indicate river systems. Map was reorganized from Voris (2000).

Assuming the translocation occurred only in an ancestral population of *H. a. agilis* and *H. agilis unko* after isolating, the geographical barriers should have served as an isolation mechanism between the subspecies with and without the mutation, though the puzzle of why three captives of *H. a. albibarbis* had the WAT is still unsolved. Such faults will also be rectified by the more refined analyses using wild-born animals. In the re-examinations, a molecular phylogenetic analysis will elucidate the time of divergence between the subspecies, and the time of translocation genesis will be estimated accordingly. Furthermore, the combined morphological, cytogenetic, and molecular evidence will provide enough information to argue for the differentiation of the three *H. agilis* subspecies.

Influence of chromosome rearrangements on population structure

Generally, heterozygosity of reciprocal translocation may result in reduced fitness. The production of multivalents at the metaphase of

meiosis I is a cause for non-disjunction and it segregates genetically stable and unstable haploid sets (gametes) due to the duplication or deletion of the translocated elements. The events theoretically produce about 50% unstable and unviable gametes (aneuploid) or lethal gametes (White 1973). In the case of *H. agilis*, the translocation created a genetic differentiation between the subspecies. The $8/9A^{Mc/ci}$ ($8c'$ morph in vanTuinen *et al.* 1999) can construct a genome set only with $9/8M^{i/ci}$ ($9'$ morph in vanTuinen *et al.* 1999) due to a meiotic genetic segregation. The genetic segregation also regulates a counterpart of the heterozygotic pair so the mechanism excludes unmatched chromosomes. *H. a. agilis* and *H. agilis unko* thus have never shown two morphs of $8A^{Mc/i}$ (previous $8c$ morph) and $8A^{M/ci}$ (previous $8c$ morph) in either the previous (vanTuinen *et al.* 1999) or the present study, but *H. a. albibarbis* had all inversions (Table 1). However, since fitness of the heterozygote for a reciprocal translocation seems to vary for different species (Lande 1979) and fertility loss in translocation heterozygotes may be minimal in *H. agilis* (vanTuinen *et al.* 1999), WAT-heterozygous gibbons should be examined for fecundity and gamete quality conscientiously.

In order to preserve rare species, it is important to know the cytogenetic background of the animal. If there is no cytogenetic makeup, there could be a risk of decreasing fecundity or loss of offspring of rare species. Benirschke & Kumamoto (1991) therefore proposed to identify the chromosomal characteristics of animals reared in zoological institutions. VanTuinen *et al.* (1999) discussed the importance of captive management of the gibbons in zoological institutions, then described how chromosomal rearrangements are good landmarks for identifying each species in most, if not in all cases. Benirschke & Kumamoto (1991) also noted that cytogenetic analysis has become an essential part of the management and conservation of captive wild animals. It was also pointed out that cytogenetic considerations are particularly important in evaluating the possible reasons for breeding failures in zoos (Ballou & Ralls 1982). In the case of translocation in gibbons, as discussed above, five assortments of chromosomes 8 and 9 were thought to be breeding failures by deletion and duplication of the long arm fragment. However, in addition to the utility of the cytogenetic data,

the existence of chromosomal polymorphisms shared among some species underscores the need to reconsider the grade of speciation of the 44-chromosome gibbon.

The three inversions occurring in chromosome 8 have been shared in the four species (Table 1), as previously reported. Particularly, Stanyon *et al.* (1987) described that the inversion variants were polymorphically observed in four species of *H. muelleri*, *H. agilis*, *H. lar*, and *H. moloch*. They suggested that the inversions shared among the four species could indicate that these taxa have only recently diverged, or are not yet fully differentiated species, with gene flow maintaining karyological uniformity. Careful investigation using wild populations of gibbons from known localities will be able to clarify whether such chromosomal rearrangements are polymorphically shared in all species of the subgenus *Hylobates*, or whether only some species have such a specific chromosomal variation. For instance, *H. pileatus* is monomorphic for 8b (present 8M^{1/c}) of the original morph (vanTuinen *et al.* 1999) and only *H. agilis* has polymorphically the whole-arm translocation (vanTuinen *et al.* 1999 and this study). Discussion related to speciation within the gibbons will be influenced by such results.

Another problem in the preservation of endangered animals is misunderstanding of taxonomy. Particularly, in the case of gibbons, frequent misidentification of *H. agilis*, *H. moloch*, and *H. muelleri* has been published in the literature and has been revealed through correspondence with zoological institutions (vanTuinen *et al.* 1999). In our experience, some individuals in zoological institutions had been previously misidentified. For instance, since females #27 and #38 (*H. agilis unko*) had black pelage and no white brow (which was black due to being housed indoors), they were misidentified as a *H. klossii* (Kloss' gibbon). In addition, due to the misidentification of pelage, a *H. agilis albibarbis* (#29) was thought to be a *H. muelleri*; #35 and #36 (*H. muelleri*) were thought to be *H. moloch*; and #39 (*H. agilis unko*) was thought to be *H. moloch*. If these misidentifications were adopted in the present study, we would have concluded that the translocation observed here was shared in four of the species that we examined, *H. agilis*, *H. muelleri*, *H. moloch* and *H. klossii*. In fact, re-identification through a blind test (judged only from photographs) revealed that

the translocation exists only in *H. agilis*, probably in two of the three subspecies (*H. a. agilis* and *H. agilis unko*). It is extremely important to know the species geographical location of wild-born individuals and the exact parentage of captive-born individuals in order to monitor species genetically and to identify the molecular phylogeny of the 44-chromosome gibbons.

Acknowledgements

We appreciate Rangers of Conservation Biology in Kalimantan for their assistance in obtaining samples from pet gibbons, and the staff members of the Ragunan Zoo, Jakarta for supplying gibbon blood samples. We also thank Drs. Y. Kawamoto, S. Kodera, H. Hori and Geri-Ann Galanti for their valuable comments and encouragement to do this study. This study was supported in part by The Japan Society for Promotion of Science Grants 08041147 and 10CE20051 (to O.T.) and 08454279 and 14405020 (to H.H.).

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