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A whole-arm translocation (WAT8/9) separating Sumatran and Bornean agile gibbons, and its evolutionary features

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Abstract

Gibbons, like orangutans, are a group of threatened Asian apes, so that genetic monitoring of each species or subspecies is a pressing need for conservation programmes. We conducted a project to take, as far as possible, samples of known origin from wild-born animals from Sumatra and Borneo (Central Kalimantan) for genetic monitoring of agile gibbons. As a result, we found a whole arm translocation between chromosomes 8 and 9 (WAT8/9) specific to Sumatran agile gibbons. Furthermore, population surveys suggested that the form with the WAT8/9 seems to be incompatible with an ancestral form, suggesting that the former might have extinguished the latter from Sumatran populations by competition. In any case, this translocation is a useful chromosomal marker for identifying Sumatran agile gibbons. Population genetic analyses with DNA showed that the molecular genetic distance between Sumatran and Bornean agile gibbons is the smallest, although the chromosomal difference is the largest. Thus, it is postulated that WAT8/9 occurred and fixed in a small population of Sumatra after migration and geographical isolation at the last glacial period, and afterwards dispersed rapidly to other populations in Sumatra as a result of the bottleneck effect and a chromosomal isolating mechanism.

Introduction

Small apes (Hylobatidae) are included in Appendix I (threatened species) of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) as endangered species, together with great apes and other monkeys (Soehartono & Mardiastuti 2002). Forest clearance has reduced their habitat, which is primary forest with tall trees. If this continues in the future, it may cause the extinction of small apes. To prevent such an undesirable outcome, conservation programmes are starting in several places. As part of these programmes, conservation genetics could be important in reducing current rates of extinction and preserving biodiversity (reviewed in Frankham *et al.* 2002). That is, genetic factors that affect extinction risk can be

determined, and the genetic management schemes required to minimize these risks can be put in place. As basic major genetic issues for starting conservation biology, evolutionary, population and quantitative genetics and taxonomy are probably the most important for the precise understanding of the biodiversity of these species.

In gibbons, hitherto, there have been problems with the identification of species or subspecies in zoological institutions. Most such captive gibbons are of unknown origin, so that identification is very difficult and is sometimes confused due to morphological similarity. Such confusion results in strange molecular phylogenetic relationships. Phylogenetic trees have also been produced showing topology such as 'trans-species polymorphisms' of mitochondrial DNA sequences in investigations using samples obtained from zoological institutions (unpublished data), as a result of misidentification or interspecific hybridization in captivity. Such indefinite data lead to confusion in genetic monitoring to obtain standard taxonomic information in conservation programmes. To avoid such problems, samples of known origin are required as an initial step.

Chromosomes of small apes are highly differentiated from those of other apes in spite of belonging to the same hominoid group. First, the genome of each subgenus of small apes is very intricately rearranged compared with humans and great apes due to numerous translocations (Jauch *et al.* 1992, Koehler *et al.* 1995, Arnold *et al.* 1996, Nie *et al.* 2001, Müller *et al.* 2003). These rearrangements are more drastic than those that distinguish human and Old World monkeys, and are a puzzle of primate chromosome evolution. However, the chromosome number is an important feature for separating the four subgenera of small apes, that is, *Bunopithecus* (2n = 38), *Hylobates* (2n = 44), *Symphalangus* (2n = 50), and *Nomascus* (2n = 52) (reviewed in Geissmann 1995). Additionally, molecular cytogenetic data indicated that the subgenus *Bunopithecus* is the most basal group of the family Hylobatidae, followed by *Hylobates*, with *Symphalangus* and *Nomascus* as the last to diverge (Müller *et al.* 2003). This result conflicts with the view from molecular phylogenetic studies with DNA sequences that depicts *Nomascus* as the most basal group of the Hylobatidae, followed by *Symphalangus*, with *Bunopithecus* and *Hylobates* as the last to diverge (Ross & Geissmann 2001). This discrepancy might occur because speciation of all extant gibbons occurred within a relatively short evolutionary time, resulting in poor correlations between genetic (DNA sequence), cytogenetic (chromosome differentiation), and morphological divergence (Müller *et al.* 2003). This can also be deduced from the fact that all six species of the subgenus *Hylobates* share the same three chromosome inversions (e.g. Stanyon *et al.* 1987, Van Tuinen *et al.* 1999). In general, chromosomal rearrangements can produce changes that are considered as unique landmarks at the divergence nodes (Müller *et al.* 2003) because chromosome changes generate reproductive isolation between populations by reducing fitness (White 1973, 1978, King 1993). Furthermore, in new models, chromosomal rearrangements reduce gene flow by suppressing genetic recombination and extending the effects of linked isolating genes (e.g. Rieseberg 2001, Navarro & Barton 2003a), and accelerate protein evolution (Navarro & Barton 2003b). These new and old models may all lead to chromosomal speciation. Since the small ape lineage has passed through a burst of translocations, processes such as meiotic drive, recombination reduction, and molecular divergence in rearranged chromosomes might have operated as valid evolutionary processes in gibbons. Therefore, chromosome markers could be very useful tools for evolutionary and/or conservation studies of gibbons.

Recently, a whole arm translocation (actually an almost whole arm translocation) between chromosomes 8 and 9 was found in the agile gibbon (*Hylobates agilis*; Van Tuinen *et al.* 1999). This translocation (named WAT8/9) seemed to be restricted to Sumatran agile gibbons (Hirai *et al.* 2003) though data were not adequate because the samples were of unknown origin. We thus conducted a research project to determine the situation in individuals of known origin because, if our postulation were correct, such information would be very useful for identifying individuals whose specific or subspecific status was uncertain from purely morphological data. This would be important for conservation programmes. This report describes their chromosome differentiation and the population structure of the variation, together with morphological identification, clustering of TSPY (testis-specific protein, Y encoded) gene

sequences, and genetic constitution of microsatellite DNA, and discusses mechanisms of formation of WAT8/9 using chromosome-painting data, features of the heterozygote, migration of the species group, and suggestions for conservation programmes.

Materials and methods

Samples, chromosome preparation and DNA extraction

Table 1 lists taxonomic identification, place of origin (if known), and morphs of chromosome 8 in the 49 gibbons used here. Identification of species and subspecies (Geissmann 1995, Marshall & Sugardjito 1986, Mootnick (2005)) was done by one of us (A.R.M.) using only information about locality (for animals of known origin) and photographs (face, profile, head, chest, back, genital tuft, hand, arm, leg and foot) taken under anaesthesia after blood sampling. Ten individuals of known origin from Sumatra and seven individuals of unknown origin, were identified as lowland agile gibbon (*Hylobates agilis unko*; UN) or highland agile gibbon (*H. agilis agilis*; AG). As the two subspecies are not clearly distinguishable in the present study, they are named UN/AG, meaning either UN or AG. Ten individuals of known origin from Borneo (Central Kalimantan) and nine individuals of unknown origin were identified as Bornean agile gibbon (*H. agilis albibarbis*; AL). Finally, ten individuals of known origin from Borneo (Central Kalimantan) and three individuals of unknown origin were identified as Müller's Bornean gibbon (*H. muelleri*; MU). Samples were placed into two classes, clarified by interview: (wild) for known origin, and (captive) for unknown origin. Distinction of known origin and unknown origin was made by interviewing the owners of the gibbons. Approximate original localities of individuals of known origin are plotted on the map (Figure 1).

The methods of anaesthesia (3–5 mg ketamine/kg body weight) and blood sampling (2 ml/kg body weight) were in accordance with the guidelines for the care and use of primates of our institute (Primate Research Institute, Kyoto University; KUPRI; Primate Research Institute, Kyoto University 2002). 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). Total DNA was extracted from whole blood using a kit (QIAamp DNA Blood Mini Kit, QIAgen). The fixed cell preparations and purified DNA were transferred to KUPRI with the permission of CITES (Export Nos. 02830/IV/SATS-LN/2003 and 08556/IV/SATS-LN/2003; Import Nos. 5020–14–0002 and 5020–15–0001) for chromosome painting analysis by fluorescence *in-situ* hybridization and for DNA analyses.

Chromosome painting analysis

According to previous data (Jauch *et al.* 1992), chromosome 8 of the subgenus *Hylobates* (44-chromosome gibbons) can be reconstructed from human chromosomes (HSACs) 16, 5, 22, 17 and 9, and *Hylobates* chromosome 9 from HSACs 17 and 9 from the top of the short arm to the bottom of the long arm, respectively. Thus HSAC painting probes 22 (CP5622-red and -green, Qbiogene), 17 (CP5617-green), and 9 (CP5605-red) were chosen for detailed analysis of the WAT8/9 rearrangement in model animals at KUPRI (Figure 2). A total of 9 µl of probe solution, consisting of 3 µl of HSAC 9-red, 3 µl of HSAC 17-green, 1.5 µl of HSAC 22-green, and 1.5 µl of HSAC 22-red, was applied to a chromosome preparation using a technique described previously (Hirai *et al.* 2003). This system can detect fragments of each HSAC by three different fluorescence colours, red for HSAC 9, green for HSAC 17, and yellow for HSAC 22. The incidence of WAT8/9 in 49 individual samples collected in Indonesia was examined using this HSAC painting system, because the three probes can detect three inversions (8a, 8b, 8c) and the WAT8/9 (8c′), though 8a and 8b were sometimes indistinguishable in some low quality chromosome samples because they differ by just a small inversion (see Van Tuinen & Ledbetter 1983, Stanyon *et al.* 1987, Hirai *et al.* 2003). Therefore, 8a and 8b were described here as 8a,b, indicating either 8a or 8b. Painting signals were observed and recorded with a digital image-analyzing system consisting of an Axiophot microscope (Zeiss)

Table 1. Origins and taxonomic and karyotypic data of agile and Müller's gibbons collected from Sumatra and Borneo (Central Kalimantan).

Number	Taxon[1]	Sex	Origin	Facility	8 morph[2]
Sumatran agile gibbons					
1	UN	Male	Wild: Pasaman, Sumatra	Pet	8a.b/8c′
2	UN	Male	Wild: Riau, Sumatra	Pet	8c′/8c′
3	UN/AG	Male	Wild: Lembah Harau Nature Reserve, Sumatra	Zoo	8c′/8c′
4	UN	Male	Wild: Solok Selatan, Sumatra	Pet	8c′/8c′
5	UN	Female	Wild: Muaralabuh, Sumatra	Pet	8c′/8c′
6	UN	Female	Wild: Ujingbatu, Sumatra	Pet	8a.b/8c′
7	UN/AG	Female	Wild: Lundar Harau, Sumatra	Pet	8c′/8c′
8	UN/AG	Female	Wild: Ujung Gaging, Pasaman Barat, Sumatra	Pet	8c′/8c′
9	UN	Female	Wild: Sijunjung, Sumatra	Pet	8c′/8c′
10	UN	Female	Wild: Perkebunan, Solok Selatan, Sumatra	Pet	8c′/8c′
11	UN	Male	Captive	Zoo	8c′/8c′
12	UN	Male	Captive	Zoo	8c′/8c′
13	UN	Male	Captive	Zoo	8c′/8c′
14	UN/AG	Male	Captive	Zoo	8a.b/8c′
15	UN	Male	Captive	Zoo	8c′/8c′
16	UN	Female	Captive	Zoo	8a.b/8c′
17	AG	Female	Captive	Zoo	8c′/8c′
Bornean agile gibbons					
18	AL	Male	Wild: Palankaraya, Borneo	Pet	8c/8c
19	AL	Male	Wild: Palankaraya, Borneo	Pet	8a.b/8a.b
20	AL	Male	Wild: Pangkalanbun, Borneo	Pet	8c/8c/
21	AL	Male	Wild: Pangkalanbun, Borneo	Pet	8a.b/8a.b
22	AL	Male	Wild: Kota Warigigin Barat, Borneo	Pet	8a.b/8c
23	AL	Male	Wild: Kota Warigigin Barat, Borneo	Pet	8a.b/8c
24	AL	Female	Wild: Tumbangjutuh, Borneo	Pet	8a.b/8c
25	AL	Female	Wild: Palankaraya, Borneo	Pet	8c/8c
26	AL	Female	Wild: Mendawai, Borneo	Pet	8c/8c
27	AL	Female	Wild: Nangabilik, Borneo	Pet	8a.b/8c
28	AL	Male	Captive	Zoo	8a.b/8a.b
29	AL	Male	Captive	Pet	8a.b/8c
30	AL	Male	Captive	Pet	8a.b/8c
31	AL	Male	Captive	Pet	8c/8c
32	AL	Female	Captive	Zoo	8a.b/8c
33	AL	Female	Captive	Pet	8c/8c
34	AL	Female	Captive	Pet	8a.b/8a.b
35	AL	Female	Captive	Pet	8c/8c
36	AL	Female	Captive	Pet	8a.b/8c
Müller's Bornean gibbons					
37	MU	Male	Wild: Buntok, Borneo	Pet	8a.b/8c
38	MU	Male	Wild: Buntok, Borneo	Pet	8c/8c
39	MU	Male	Wild: Temiang Layang, Kabupaten, Barito Timur, Borneo	Pet	8a.b/8c
40	MU	Male	Wild: Muara Teweh, Borneo	Pet	8a.b/8a.b
41	MU	Female	Wild: Buntok, Borneo	Pet	8c/8c
42	MU	Female	Wild: Buntok, Borneo	Pet	8a.b/8a.b
43	MU	Female	Wild: Buntok, Borneo	Pet	8a.b/8c
44	MU	Female	Wild: Muara Teweh, Borneo	Pet	8a.b/8a.b
45	MU	Female	Wild: Muara Teweh, Borneo	Pet	8a.b/8a.b
46	MU	Female	Wild: Ampah, Buntok, Borneo	Pet	8a.b/8c
47	MU	Male	Captive	Pet	8c/8c
48	MU	Male	Captive	Zoo	8a.b/8c
49	MU	Female	Captive	Pet	8a.b/8c

[1]UN, *Hylobates agilis unko*; AG, *H. agilis agilis*; AL, *H. agilis albibarbis*; MU, *H. muelleri*. [2]morph of chromosome 8. 8a, 8b, 8c, morphs with different inversion. 8c′, karyotype occurred by WAT8/9 rearrangement.

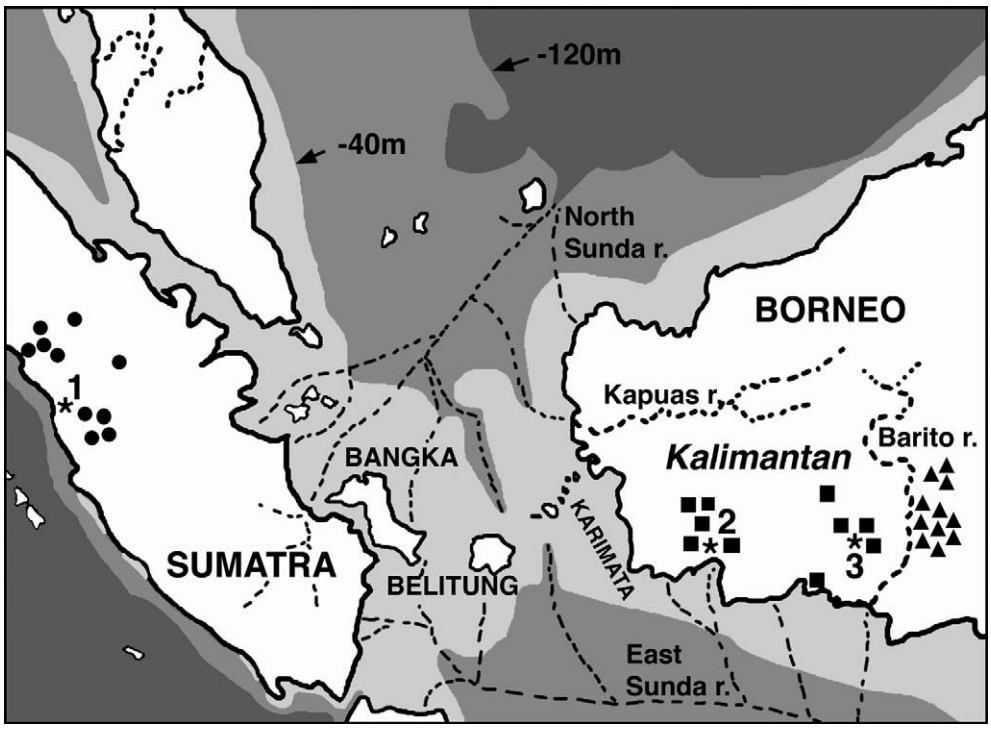

Figure 1. Partial map of Indonesia showing original localities of gibbons of known origin in the islands of Sumatra and Borneo, and historical sea level changes of Sunda land at the last glacial period. Numbered asterisks indicate principal cities near the sampling sites: 1, Padang, Sumatra; 2, Pangkalanbuun, Central Kalimantan; and 3, Palangkaraya, Central Kalimantan. Solid circles show original localities (wild in Table 1) of Sumatran agile gibbons (UN/AG), solid squares Bornean agile gibbons (AL), and solid triangles Müller's Bornean gibbons (MU). Colour gradation from white to dark grey indicates change of sea level from the last glacial period to the present: white, present-day land; light grey, 40 m below present-day level; grey, 120 m below present-day level; dark grey, sea at the last glacial period. Dotted lines are river systems.

with Cool SNAP-HQ CCD camera (Photometrics) controlled by IPLab software (Scanalystics) running in a Macintosh computer (G4). The image data were analyzed with Adobe Photoshop software to determine the mechanism of the chromosome rearrangements.

DNA analyses

To clarify divergence within agile gibbon species, molecular phylogenetic analyses of clustering of TSPY sequences were made, and genetic divergence of microsatellite DNA loci was analysed. Since these DNA analyses were used just for showing the genetic background to support the chromosomal results in the present study, however, the details of techniques and data will be described elsewhere. Briefly, TSPY sequences were obtained from genomic DNA extracted from blood samples of nine males of UN/AG, eight males of AL, and six males of MU by PCR amplification using primers described previously (Kim *et al.* 1996). A network of TSPY haplotypes was drawn with the TCS program (Clement *et al.* 2000) using sequence alignment data analysed using CLUSTAL X 1.81 (Thompson *et al.* 1997). To analyse the genetic relationship between the three groups, 17 UN/AG, 18 AL, and 11 MU were examined with fragment size variation in 12 loci of human microsatellite DNA. Analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) was conducted with ARLEQUIN (Schneider *et al.* 2000), and F_{ST} distance was calculated for measuring the extent of genetic distance between groups with variance of genotypic frequencies in 195 independent alleles.

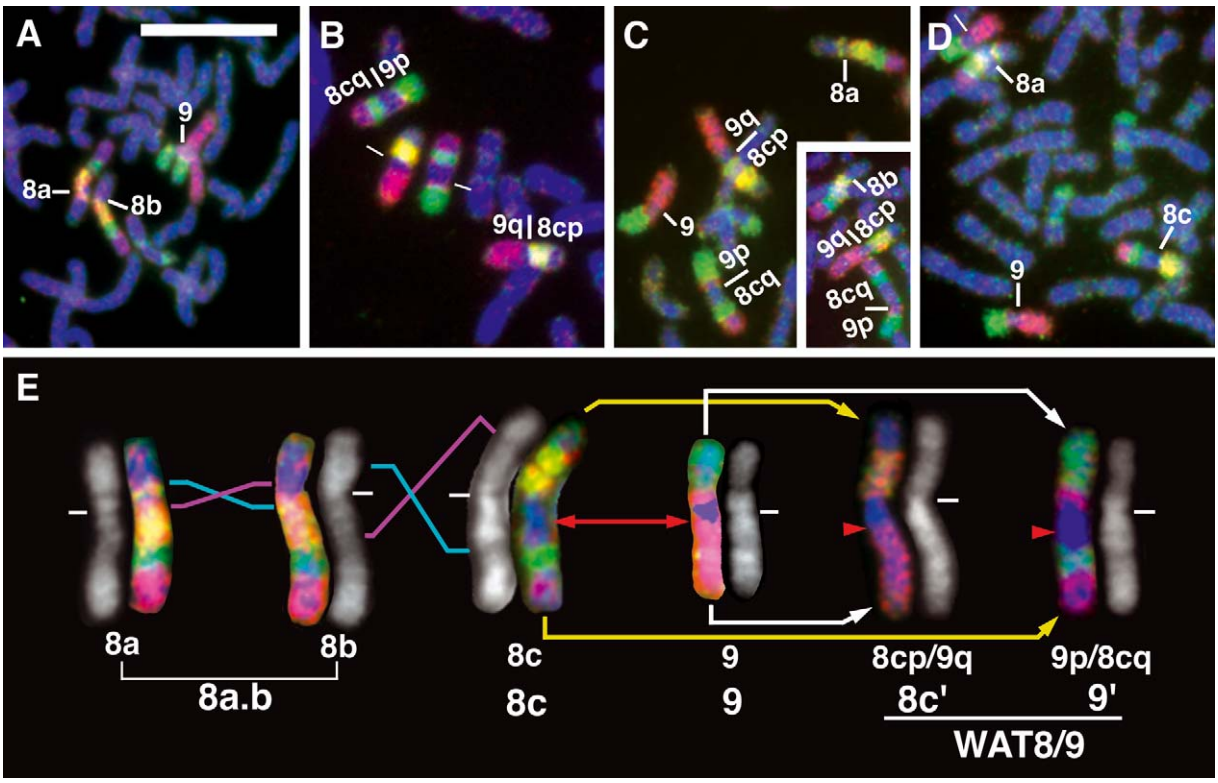

Figure 2. Chromosome painting analysis of agile gibbons using human chromosome painting probes HSACs 9 (red), 17 (green), and 22 (yellow). (**A**) Partial metaphase spread with chromosomes 8a, 8b, 9 and 9. (**B**) Partial metaphase spread with chromosomes 8c′ (=8cp/9q), 8c′, 9′ (= 9p/8cq), and 9′. (**C**) Partial metaphase spread with chromosomes 8a, 8c′ (= 8cp/9q), 9, and 9′ (= 9p/8cq). (**C**) **Inset**, partial metaphase spread with chromosomes 8b, 8c′ (= 8cp/9q), and 9′ (= 9p/8cq). (**D**) Partial metaphase spread with chromosomes 8a, 8c, 9, and 9. (**E**) Mechanisms of alterations in chromosomes 8 and 9. p, chromosome short arm; q, chromosome long arm. The small white bars indicate the centromeres. Blue and pink lines indicate breakage points of inversions. Red arrows and arrowheads show breakage points of WAT8/9 that produced 8c′ and 9′. White and yellow arrows indicate locations transferred from original regions. For details see text.

Results and discussion

Formation of WAT8/9

A family of four agile gibbons – the mother (Eve), father (Mamy), and two sons (Raja and Tsuyoshi), who live at KUPRI – was examined as a model case of chromosome differentiation related to WAT8/9 formation, using chromosome painting (Figure 2). Eve is heterozygous for two types of inversions of chromosome 8, 8a and 8b (Figure 2A). Mamy is homozygous for WAT8/9, 8cp/9q and 9p/8cq (Figure 2B). Raja is heterozygous for 8a and 8cp/9q and 9 and 9p/8cq in chromosomes 8 and 9 (Figure 2C). Tsuyoshi is heterozygous for 8b and 8cp/9q and 9 and 9p/8cq in chromosomes 8 and 9 (Figure 2C inset). Finally, a representative individual Bornean agile gibbon shows a heterozygous pair of chromosome 8, 8a and 8c (Figure 2D). These examples demonstrate manifestation of chromosome painting analysis of all rearranged chromosomes 8 and 9 in agile gibbons.

Chromosomes 8a, 8b, and 8c are identical to the three inversion morphs, respectively, that previous studies (Van Tuinen & Ledbetter 1983; Stanyon *et al.* 1987) designated with G-banding analysis. Chromosomes 8cp/9q and 9p/8cq that had undergone the WAT8/9 rearrangement are identical to the 8c′ and 9′ morphs that were discovered with G-banding (van Tuinen *et al.* 1999), and to $8/9A^{Mc/ct}$ and $9/8^{Mi/ci}$ designated with a

C-banding technique (Hirai *et al.* 2003). Chromosomes 8a and 8b are differentiated by a small pericentric inversion, so that they were sometimes confused during the survey of chromosome alterations in samples from Indonesia. Therefore 8a and 8b were lumped together as 8a,b in the description of the survey of 49 gibbons (see the bottom of Figure 2E for clarification of the nomenclature).

Incidence of WAT8/9

Observation of a total of 49 individuals showed a remarkable karyotypic difference between Sumatran and Bornean gibbons (Table 1). Ten wild-born gibbons of known origin and seven captives of unknown origin of the UN/AG subspecies comprised 13 homozygotes of WAT8/9 (8c′/8c′) and four heterozygotes of non-WAT8/9 and WAT8/9 (8a,b/8c′) in chromosome 8, whose frequency of chromosome morph was 8a,b (11.8%), 8c (0%), and 8c′ (88.2%). However, AL showed only inversion polymorphisms of 8a,b (42.1%) and 8c (57.8%). MU also showed polymorphisms of 8a,b (53.8%) and 8c (46.2%) similar to those found in AL (Table 1). In AL and MU, WAT8/9 was not found.

As described above, four heterozygotes of 8a,b and 8c′ were found in the UN/AG samples. In previous studies, another four (van Tuinen *et al.* 1999) and five (Hirai *et al.* 2003) heterozygotes of 8a,b/8c′ were found, which were believed to be the first filial generation (F1) in both wild- and captive-born animals. It should be noted that all 13 heterozygotes were 8a,b/8c′, and the other expected combination of 8c/8c′ has never been found so far. Random breeding of pairs in wild populations and zoological institutions would have been expected to produce not only 8a,b/8c′ heterozygotes but also 8c/8c′ ones. Nevertheless, the apparent absence of the heterozygote 8c/8c′ suggests that any isolation mechanism must have operated on the heterozygotes. Consideration of the postulated pairing structure in meiosis suggests that the absence of 8c/8c′ heterozygotes would be unexpected (see Figure 3). Quadrivalent assortment of 8a,b, 8c′ , 9, and 9′ (Figure 3B, left) has eight or nine separated pairing segments, while that of 8c, 8c′, 9 and 9′ (Figure 3B, right) has six separated pairing segments. Thus the former set (Figure 3B, left) is more complicated and disadvantageous at the cytogenetic level and expected to be less viable than the latter set (Figure 3B, right). Despite its higher viability, however, the latter heterozygotic assortment has in fact never been found so far. Van Tuinen *et al.* (1999) clearly showed the cross-configuration of the synaptonemal complex in a quadrivalent of 8a, 8c′, 9, and 9′ and viable offspring up to F4 of the heterozygous carriers. Judging from these observations, the latter set of 8c and 8c′ may have a certain genetic disorder based on 'latent structural differentiation' which cannot develop into mature gametes (postmating isolation), whose mechanism is unknown. In any case, an unknown mechanism of postmating isolation may exist in the pairing of 8c and 8c′. The unknown antagonistic mechanism between 8c and 8c′ may have extinguished the 8c morph from the Sumatran populations after the WAT8/9 translocation occurred. The 8c chromosome is thus not found in the Sumatran subspecies (UN/AG) at the present time. Populations of AL and MU that are species or subspecies derived from the same ancestral population as UN/AG, which will be mentioned below, consist of 22 (35.5%) 8a,b chromosomes and 40 (64.5%) 8c and 24 (52.2%) 8a,b and 22 (47.8%) 8c (data combined Hirai *et al.* 2003 and the present study), respectively. Thus, UN/AG populations also might have had 8c chromosome before the WAT8/9 occurred. If 8c had not originally been in the population, then the WAT8/9 translocation could not have been produced. That is, 8c′ could be incompatible with 8c. This mechanism may promote prompt fixation of 8c′ in Sumatran populations. This chromosome trait will need to be considered in management plans for conservation programmes.

Molecular genetic relationship between Sumatran and Bornean gibbons

Based on cluster analysis using TSPY gene sequences, the relationship between UN/AG, AL and MU was drawn as a network in Figure 4. UN/AG and AL are distinct from each other with 2–7 base pairs difference in the sequence, and both of them are distinct from MU with 6–10 base pairs difference (Figure 4). The TSPY divergence was confirmed by a genetic distance analysis with microsatellite DNA fragments. The F_{ST} value was calculated and a 5000 times

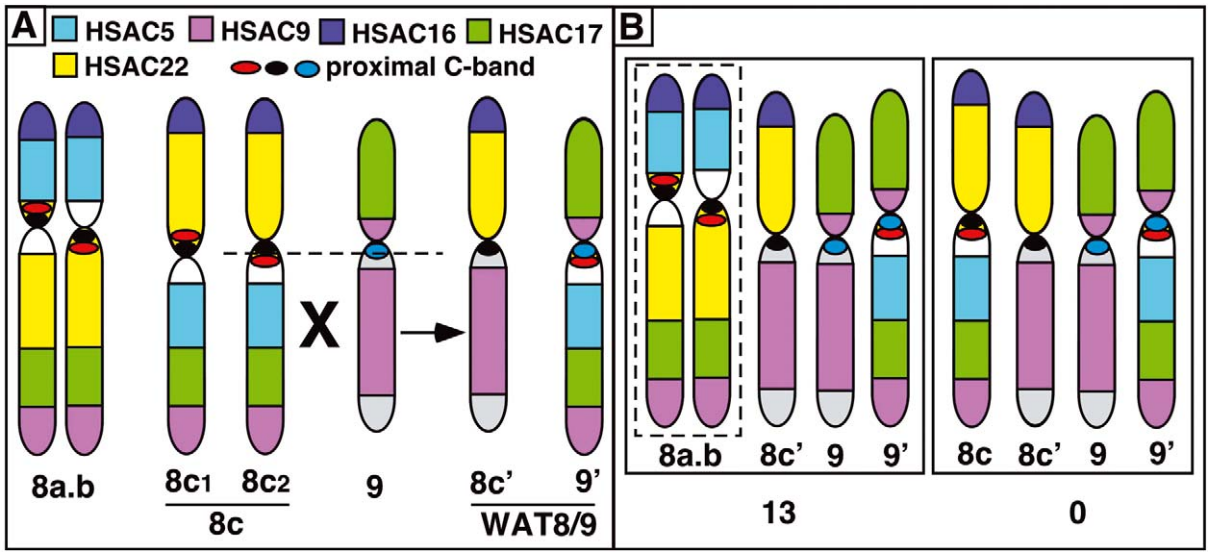

Figure 3. Schematic pathway of chromosome changes in agile gibbons (**A**) and expected heterozygotic patterns (**B**). WAT8/9 formation was confirmed by data from the present and previous painting analyses (Jauch *et al.* 1992, van Tuinen *et al.* 1999, Hirai *et al.* 2003) and C-banding analysis (Hirai *et al.* 2003). 8c is divided into two subtypes according to centromere location, whether moved by a centromere shift or a small pericentric inversion in the block of heterochromatin (Hirai *et al.* 2003). (**A**) Two quadrivalent assortment patterns in the heterozygote, consisting of WAT8/9 and non-WAT8/9 chromosomes, are expected based on chromosome variations (**B**). Numbers at the bottom of (**B**) are numbers of F1 heterozygotes observed in the present and previous (van Tuinen *et al.* 1999, Hirai *et al.* 2003) studies. In chromosomes 8, 9 and their derived ones, segments with no paint hybridization are coloured in white or grey. For details see text.

permutation test for its significance was performed by AMOVA using data from 195 alleles of 12 microsatellite loci. The AMOVA result indicated genetic difference among the three groups ($F_{ST} = 0.053$, $p < 0.001$). Further, the significant pairwise F_{ST} value ($p < 0.001$) obtained between UN/AG and AL was 0.0314; between AL and MU 0.0558, and between UN/AG and MU, it was 0.0849. They thus are genetically distinct from each other. Replacing TSPY haplotypes with karyotypes gives a remarkable chromosomal differentiation between gibbons from Sumatra and Borneo (see Figure 4).

Both of the DNA investigations revealed that UN/AG and AL are much more closely related to each other than to other gibbons. However, karyotypes of UN/AG and AL showed the most pronounced difference, while AL and MU, which are most different at the DNA level, showed similar polymorphism for three inversions. This allows us to postulate that isolation between UN/AG and AL by the Karimata Strait occurred more recently than that between other populations, and that the WAT8/9 translocation occurred only in UN/AG after geographical isolation. If the data from the present study are assumed to be a symbolic representation of UN/AG, WAT8/9 should have broken through the severe evolutionary bottleneck and afterward promptly dispersed to and become established in other populations of Sumatra. The explosion of the Toba Volcano about 74 000 years ago would be a cause of ecosystem change giving rise to the bottleneck effect, as discussed in previous studies (e.g. Muir *et al.* 2000). The present study at least reveals that gene flow between the two islands might have not occurred after geographical isolation.

From the results of the DNA divergence studies, Bornean gibbons (AL and MU) are assumed to have originated from at least two migration events from Sumatra to Borneo during the last glacial period, though the data are inconclusive. DNA differences between UN/AG, AL, and MU show at least two step differentiations, and the sea level over the Sunda Shelf has changed frequently, ranging from 40 m to 120 m lower than at present (see

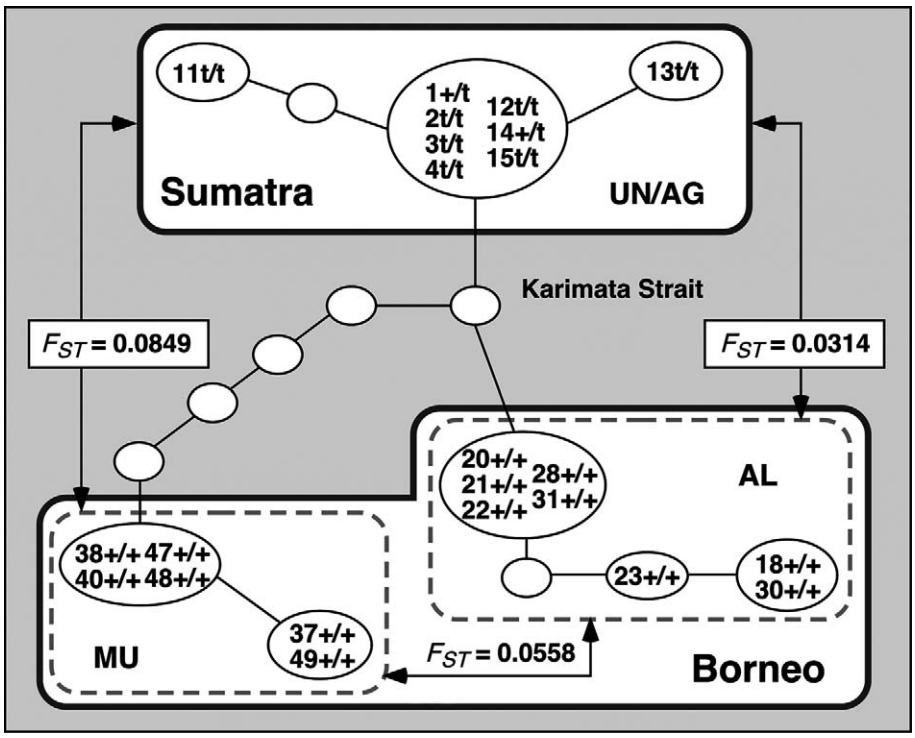

Figure 4. Schematic relationships between Sumatran (UN/AG) and Bornean (AL) agile gibbons, and Müller's Bornean gibbons (MU), showing the network of TSPY gene sequences, genetic distance between groups (arrows) calculated with alleles of microsatellite DNA loci, and incidence of WAT8/9 of males. An interval bar between ovals indicates one base pair substitution. Blank ovals indicate intermediate haplotypes undiscovered so far. Numbers refer to the individuals listed in Table 1. t, WAT8/9. +, non-WAT8/9. For details see text.

Figure 1; Mackinnon *et al.* 1997, Voris 2000, Whitten *et al.* 2000). When the sea was 40 m to 120 m below present-day sea levels, land bridges would have been formed as a result of geographical and climatic changes, resulting in island vegetation corridors (Hantoro 1997, Meijaard 2003). In particular, the connection between Sumatra and Borneo appears to have persisted longest through the land bridges via Bangka, Belitung, and the Karimata Islands, because they were still connected even when the sea level was only 40 m below present-day sea levels. Moreover, because it is postulated that there were comparatively large rivers (North and East Sunda rivers) in the Sunda land between Sumatra, Borneo and Java, this route is most plausible for the parsimonious migration event (reviewed in Voris 2000; see Figure 1). At least, isolation between Sumatran and Bornean agile gibbons can be postulated to have occurred at this period. The route was also taken by the more recent migration of orangutans (e.g. Muir *et al.* 2000, Warren *et al.* 2001). The precise migration time will be estimated by mitochondrial DNA analyses that will be reported elsewhere. Further, samples from agile gibbons from the Malay peninsula and south eastern Sumatra will elucidate more precisely the deductions made in the present study.

Perspective and conclusion

The present study, using gibbons of known origin, has clearly revealed that the three groups of Sumatran and Bornean gibbons are significantly diverged from each other at the levels of DNA, chromosomes and morphology, though the distinction between the subspecies (UN and AG) of Sumatran agile gibbons is not clear. Groves (2001) lumped the two Sumatran subspecies into a single

species (*H. agilis*) and classified Bornean agile gibbons (AL) as a different species, white-bearded gibbon (*H. albibarbis*) from Sumatran agile gibbons and Müller's Bornean gibbons (MU). The taxonomic question will be discussed elsewhere, together with new molecular data on the taxa. In any case, the data obtained here could be very useful as genetic markers for identifying species or subspecies that would be indispensable to conservation programmes. The WAT8/9 rearrangement is a remarkable chromosomal marker for separating Sumatran and Bornean agile gibbons. If it is correct that there is an isolating mechanism between 8c′ and 8c, then Sumatran and Bornean agile gibbons should be separated in captive populations, and not released into a wild population different from where they originated, because the 8c variant would be replaced with 8c′ carrying the WAT8/9 rearrangement. Otherwise, a human artificial bias may be given to the natural traits of the wild populations. Accordingly, a check on the chromosome constitution is required for conservation programmes of Sumatran and Bornean agile gibbons, as well as of Sumatran and Bornean orangutans, which have differences in chromosome 2 and the Y chromosome (Seuánez *et al.* 1979, Schempp *et al.* 1993).

The present study also suggested that samples of known origin should be regarded as essential for studying phylogenetic evolution or for genetic monitoring of standard species or subspecies. In our experience, some captive gibbons of unknown origin showed a mixture of traits of Sumatran (UN/AG) and Bornean (AL) agile gibbons and Müller's Bornean gibbons (MU). For example, one male gibbon showed AL type of morphology, UN/AG type of TSPY, AL type of mitochondrial DNA, and AL or MU type of chromosomes. Another male showed AL type of morphology, MU type of TSPY, AL type of mitochondrial DNA, and AL or MU type of chromosomes. The origin of such cases is difficult to understand due to insufficient knowledge of their history. Other gibbon species also need to be standardized, using samples of known origin, with morphological, genetic and ecological methods to make clear more precisely what are the evolutionarily significant units (ESUs) (e.g. Crandall *et al.* 2000) of each species or subspecies for future conservation programmes of gibbons.

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