

Construction, Characterization, and Chromosomal Mapping of a Fosmid Library of the White-Cheeked Gibbon (*Nomascus leucogenys*)

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Gibbons have experienced extensive karyotype rearrangements during evolution and represent an ideal model for studying the underlying molecular mechanism of evolutionary chromosomal rearrangements. It is anticipated that the cloning and sequence characterization of evolutionary chromosomal breakpoints will provide vital insights into the molecular force that has driven such a radical karyotype reshuffle in gibbons. We constructed and characterized a high-quality fosmid library of the white-cheeked gibbon (*Nomascus leucogenys*) containing 192,000 non-redundant clones with an average insert size of 38 kb and 2.5-fold genome coverage. By end sequencing of 100 randomly selected fosmid clones, we generated 196 sequence tags for the library. These end-sequenced fosmid clones were then mapped onto the chromosomes of the white-cheeked gibbon by fluorescence *in situ* hybridization, and no spurious chimeric clone was detected. BLAST search against the human genome showed a good correlation between the number of hit clones and the number of chromosomes, an indication of unbiased chromosomal distribution of the fosmid library. The chromosomal distribution of the mapped clones is also consistent with the BLAST search result against human and white-cheeked gibbon genomes. The fosmid library and the mapped clones will serve as a valuable resource for further studying gibbons' chromosomal rearrangements and the underlying molecular mechanism as well as for comparative genomic study in the lesser apes.

Key words: fosmid library, white-cheeked gibbon, chromosomal mapping, fluorescence *in situ* hybridization

Introduction

Gibbons (the lesser apes, genus *Hylobates*, family Hylobatidae) constitute a sister group to the great apes (Pongidae) and humans (Hominidae) (1). The four subgenera (*Bunopithecus*, *Hylobates*, *Symphalangus*, and *Nomascus*) of gibbons recognized by classical taxonomic criteria have been further characterized with four unique karyotypes and diploid numbers (2–4). Although gibbons diverged from the lineage leading to the great apes about 20 million years ago, they differ from other primates and most mammals in having an exceptionally high rate of chromosomal rearrangement (5–15). Recent chromosome painting studies

have shown that each human autosome is homologous to 2–7 segments in the genomes of gibbons. The 22 human autosomal painting probes detected 51, 60, 63–67, and 62 conserved segments in the genomes of *Hylobates lar* (2n=44), *Symphalangus syndactylus* (2n=50), *Nomascus concolor* (2n=52), and *Hylobates hoolock* (2n=38), respectively (8–10, 12). At least 80 synteny breakpoints on both human and gibbon chromosomes have been so far only assigned to chromosomal bands, which usually means a resolution of about 5–10 Mb (13, 14, 16–18). However, the characterizations of evolutionary breakpoints at the molecular level by DNA sequencing are needed to unveil the underlying genetic mechanisms that resulted in such radical rearrangements (19).

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Sequence comparison between human chromosome 19 and its mouse homologues revealed that specific DNA motifs such as clustered gene families or an unusually high concentration of L1 and LTR repeat DNA exist at the synteny breakpoints (20). Comparing the human genome with the chimpanzee genome, the pericentric inversion breakpoints were found to be rich not only in segmental duplications and low copy repeats, but also in high copy repeats and inverted segmental duplication (21–27). These results provided supports for the hypothesis that non-allelic homologous recombination between these duplicated and/or repeated sequences has played an important role in mammalian genome rearrangements (28).

Compared with human and other primates, gibbons have experienced extensive karyotype rearrangements during evolution and represent a paradigm for studying the underlying molecular mechanism of evolutionary chromosomal rearrangements. Recently, the arrangement of synteny-conserved blocks in the white-cheeked gibbon (*N. leucogenys*) genome with respect to the human genome has been refined by gibbon bacterial artificial chromosome (BAC) clone (CHORI-271 BAC library) end sequencing and clone fluorescence *in situ* hybridization (FISH) mapping analysis (29). However, the ability to detect smaller rearrangement events is limited by the insert size of the BAC vector. Alternative libraries with a smaller insert size such as the fosmid library are needed to uncover even smaller events occurred during the evolution of gibbons. In 2005, Tuzun *et al* (30) detected the intermediate-sized structural variants (inversions, deletions, and insertions) and the breakpoint characterization at the molecular level by comparing the human genome reference sequences with fosmid paired end sequences, which may provide a fine-scale structural variation map of the human genome and the requisite sequence precision for subsequent genetic studies of human diseases. Their results indicated the advantage of using fosmid library to detect smaller chromosomal rearrangements.

Here we describe the construction and characterization of a white-cheeked gibbon genomic library in a fosmid vector for further unveiling the molecular mechanisms of the high-rate genomic rearrangements in gibbons. The white-cheeked gibbon, known to be one species of *Nomascus* ($2n=52$), was chosen for library construction for two reasons. Firstly, cytogenetic studies have shown that the white-cheeked gibbon had a striking degree of karyotypic changes during its evolution (8–14, 18, 31). Comparative paint-

ing with human probes shows that the white-cheeked gibbon has the most rearranged genome in gibbons (10, 12). Secondly, the white-cheeked gibbon is one of the rarest and most endangered primates in the world because of the largely destructed habitat (32), making the white-cheeked gibbon genomic library also a valuable resource for conservation genetics study of this endangered species.

Results and Discussion

Library construction

To generate a fosmid library, genomic DNA was extracted from the lymphoblast cell line (KCB 99002) of a male Yunnan white-cheeked gibbon with size fractionized by field inversion gel electrophoresis (FIGE), and then was ligated with the fosmid vector pCC1FOS. In total, 192,000 non-redundant fosmid clones were picked up and arrayed into 500 384-well microtiter plates. The average insert size was estimated to be 38 kb (range: 33–43 kb) by testing 146 randomly selected fosmid clones through pulse field gel electrophoresis (PFGE) (Figure 1). Therefore, the total size of the white-cheeked gibbon fosmid library is estimated to be 7,296 Mb ($192,000 \times 38$ kb). According to the Animal Genomic Size Database (<http://www.genomesize.com/mammals.htm>), the haploid C value of the white-cheeked gibbon is 3.40, assuming a similar genome size with human (3.50), thus the fosmid library of the white-cheeked gibbon has at least 2.5-fold coverage of the white-cheeked gibbon genome.

Fosmid clone end sequencing and BLAST search analysis

One hundred randomly picked fosmid clones were sequenced using the T7 primer for 5' ends and the pCC1TM/pEpiFOSTM RP-2 primer for 3' ends in order to generate the sequence tags and value the chromosomal distribution of the fosmid clones. After trimming the vector sequences, the sequences of two ends (450 bp each on average) were subjected to BLAST search against the human genome. As a result, 97 fosmid clones have BLAST hits of both sequence ends anchored on the same chromosomes. The interval between paired sequenced ends from all these clones is less than 40 kb, while the remaining three clones (172B8, 171E1, and 180D9) show BLAST results resembled to those of repetitive elements. Based on the

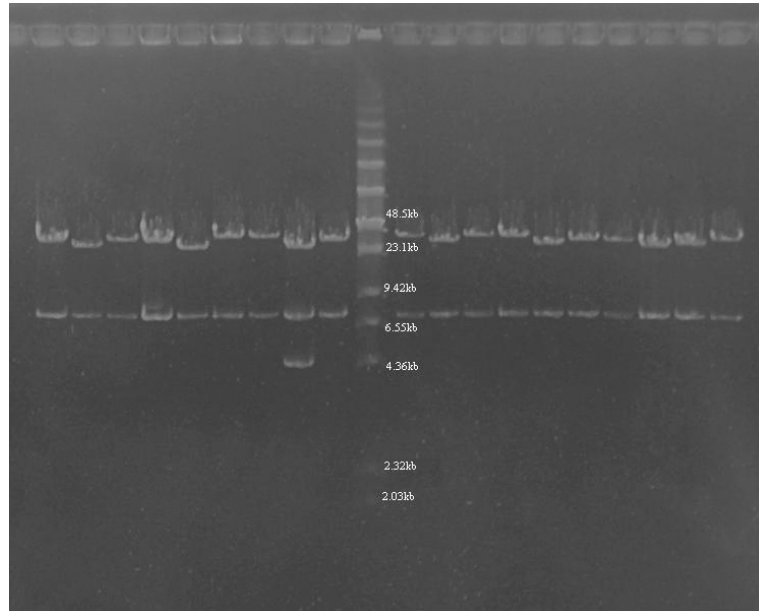


Fig. 1 The electrophoretogram of PFGE for insert size testing. There were 19 fosmid clones tested, and the low-range molecular weight marker was used as size standard in the middle of lanes. The vector band is about 7.0 kb.

analysis of the 97 unique sequences, the sequence similarity between human and white-cheeked gibbon is estimated to be 95% on average. These results reveal a very low proportion of chimeric clones in the library. In addition, the sequenced ends distributed across every chromosome of the white-cheeked gibbon except chromosomes 19 and 20, and showed a good correlation between the anchored clone number and the chromosome size (Figure 2), indicating an unbiased chromosomal distribution of the fosmid clones in the current library. The 196 sequence tags have been deposited in GenBank under Accession No. ER987117–ER987312.

FISH analysis

The 100 end-sequenced fosmid clones were also mapped onto the chromosomes of the white-cheeked gibbon by FISH. The chromosomal assignments of all fosmid clones that gave unique signals were summarized on a G-banded karyotype of the white-cheeked gibbon (Figure 2) and FISH examples are shown in Figure 3. The results of BLAST search and FISH mapping of 100 fosmid clones are listed in Table 1. Among the 97 end-sequenced fosmid clones that were also anchored on one particular human chromosome, 90 fosmid clones had unique chromosomal locations in the white-cheeked gibbon; 7 clones (47Q1, 52G1, 56O11, 66F5, 177P20, 247M1, and 399Q4) had multiple hybridization signals that “painted” the heterochromatin regions of the white-cheeked gib-

bon chromosomes. No hybridization signal indicative of chimeric clones was observed. To double-check the end sequencing results, 35 end-sequenced fosmid clones were randomly chosen and mapped onto human chromosomes by FISH (see Figure 3C and D for example). In consistent with the end sequencing data, all of them were mapped onto the right corresponding human chromosomes. Based on the relationships of synteny conservation between human and white-cheeked gibbon established by chromosome painting (14), most of the fosmid clones that hit specific human chromosomes were hybridized onto the corresponding white-cheeked gibbon chromosomes. Five clones (56I8, 182I1, 190C3, 210J1, and 280F10) gave signals on white-cheeked gibbon chromosomes that are homologous to human chromosome segments previously not detected by chromosome painting. It is likely that such subtle chromosome rearrangements could have escaped detection by cross-species chromosome painting.

The three fosmid clones mapped onto the heterochromatic regions

Among the 100 end-sequenced fosmid clones, BLAST results showed that three clones (172B8, 171E1, and 180D9) contain repetitive sequences. FISH mapping results also indicate that these three fosmid clones had hybridization signals at the heterochromatic regions of the white-cheeked gibbon chromosomes: 172B8 gave

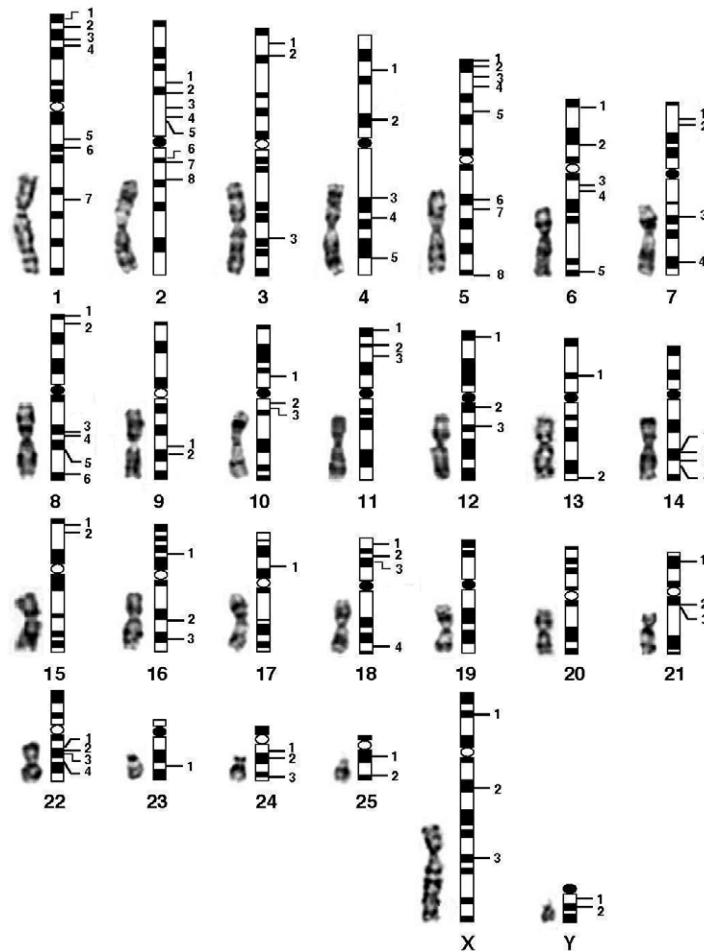


Fig. 2 The G-banded karyotype of a male white-cheeked gibbon and the ideogram with the mapped fosmid clones. The clone ID of each mapped fosmid is given in Table 1.

telomeric signals on all but two chromosomes (Chr. 16 and 25) and on the sub-centromeric regions of the short arms of chromosomes 8 and 11 (Figure 3E); 171E1 hybridized to the telomeric regions of all but three chromosomes (Chr. 11, 19 and 22), the centromeric regions of all but one chromosome (Chr. 8), and some interstitial chromosomal regions of chromosomes 3, 5, 8, and 11 (Figure 3F); 180D9 painted the white-cheeked gibbon centromeric regions of chromosomes 1, 3, 5–9, 15, 19, 22–24, and Y (Figure 3G). The single-color FISH results suggest that the insert DNA of the clones 172B8, 171E1, and 180D9 could contain overlapped regions. To elucidate the relationship of these three clones, we labeled them with biotin-16-dUTP for 172B8, FITC-12-dUTP for 180D9, and SpectrumOrange-dUTP for 171E1, respectively, and hybridized them simultaneously onto gibbon metaphases. The multi-color FISH results showed that clones 172B8 and 171E1 had overlapped signals at the telomeric regions of all but eight chro-

somes (Chr. 11, 16, 19, 22–25, and Y) and at the short arms of chromosomes 3, 8, and 11; 171E1 and 180D9 had overlapped signals at the centromeric regions of chromosomes 1, 3, 5–7, 9, 15, 17, 19, 22–24, and Y (Figure 3H). The summary of the hybridization patterns of the three clones is shown in Figure 4.

We also mapped these three clones onto the metaphase chromosomes of the white-browed gibbon, human, and macaque. The results showed that 171E1 gave no hybridization signal on the chromosomes of these three different species, 172B8 gave special signals on metaphases of these three different species, while 180D9 gave some centromeric signals on chromosomes of these three different species. Therefore, 171E1 probably contains white-cheeked gibbon-specific repetitive sequence(s). As for 172B8, the distribution of hybridization signals in the white-browed gibbon on the centromeric or paracentromeric regions of chromosomes was different from those on white-cheeked gibbon chromosomes. Based on the

Table 1 BLAST search and FISH mapping results of 100 fosmid clones

Clone ID	Human Chr. No.	Gibbon Chr. No.	Clone ID	Human Chr. No.	Gibbon Chr. No.
307H7	14q24.3	1p-1	159D7	19	10q-3
20F9	9	1p-2	282A1	20p12.1-13	11p-1
56B7	9p22.1-23	1p-3	60K3	12q	11p-2
66A3	9	1p-4	59O1	12q	11p-3
68B8	6	1q-5	04K20	1	12p-1
82E1	6q21	1q-6	32A5	1	12q-2
210J1	2q	1q-7	241N2	1	12q-3
66I3	5	2p-1	198C7	20	13p-1
47C4	5	2p-2	08H15	7	13q-2
71B3	16	2p-3	37K18	2	14q-1
182F9	16	2p-4	205I9	2p21-2p22	14q-2
61H5	16	2p-5	99O3	2	14q-3
177A2	16	2q-6	111M6	2	14q-4
111B2	16	2q-7	44C20	11q	15p-1
171C1	5	2q-8	71P24	11	15p-2
46D3	10	3p-1	22D2	8	16p-1
196B5	6q24.3-25.3	3p-2	60G1	8	16q-2
06G6	6q24	3q-3	172A9	8q	16q-3
82O1	18	4p-1	26M4	7p	17p-1
220L2	18	4p-2	203A1	6	18p-1
190H24	11	4q-3	01A3	10	18p-2
200C7	3	4q-4	19O23	5	18q-3
206H23	3	4q-5	69L1	16	18q-4
47B14	1	5p-1	51A3	3	21p-1
55P2	1	5q-2	77K2	3	21q-2
310D24	1	5p-3	10M6	3	21q-3
16F9	1	5p-4	20D3	14	22q-1
67M9	1p31.3-32.3	5p-5	120N7	14	22q-2
222J1	13	5q-6	320G1	14	22q-3
101F10	13	5q-7	282A10	14	22q-4
96L4	13	5q-8	190C3	9q32-34.11	23q-1
180A14	22q12.3-13.2	6p-1	88H4	1	24q-1
208F10	12	6p-2	106L1	1p36.21-36.32	24q-2
56I8	8	6q-3	203B7	1p35.1-36	24q-3
182I1	12	6q-4	56D8	21q22.2	25q-1
66M1	15	6q-5	123N1	21	25q-2
207L24	22	7p-1	300L3	Xp21.3-22.13	Xp-1
236H6	4	7p-2	07K1	Xq21.1-21.33	Xq-2 and Yp-2
306E1	4	7q-3	261G3	X	Xq-3
289N10	4	7q-4	82H4	Y	Yq-1
191K6	6q13-15	8p-1	47Q1*	10	*
250E1	1	8p-2	52G1*	9	*
130I9	9	8q-3	56O11*	4	*
266E	9	8p-4	66F5*	1	*
320B	9	8q-5	177P20*	6p12.3-21.2	*
490N7	9	8q-6	247M1*	12q	*
02B24	4	9q-1	399Q4*	12	*
133P14	1	9q-2	172B8#	#	#
218K10	12	10p-1	171E1#	#	#
174C17	19	10q-2	180D9#	#	#

*Could not map the unique signal because of the heterochromosome noise. #The three clones (172B8, 171E1, and 180D9) have no special unique location in human chromosomes and contain repetitive sequences in white-cheeked gibbon chromosomes (see the text and Figure 3 for detail).

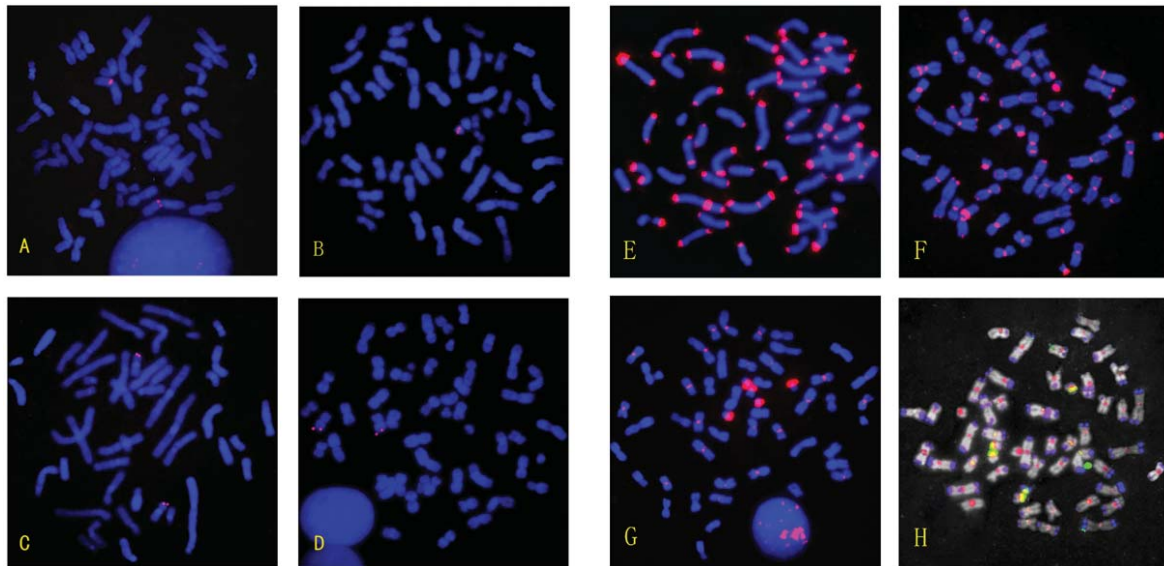


Fig. 3 Examples of FISH mapping results of fosmid clones. **A.** Fosmid clone 71B3 with a pair of signals on the short arm of chromosome 2 of the white-cheeked gibbon. **B.** Fosmid clone 82H4 hybridized onto the Y chromosome of the white-cheeked gibbon. **C.** Fosmid clone 69L1 hybridized onto the distal regions of the short arm of human chromosome 16. **D.** Fosmid clone 69L1 hybridized onto the distal regions of the long arm of chromosome 18 of the white-cheeked gibbon. **E.** Fosmid clone 172B8 with telomeric signals on chromosomes 1–15, 17–24, X, and Y, and near to the centromeric regions of the short arms of chromosomes 8 and 11 of the white-cheeked gibbon. **F.** Fosmid clone 171E1 hybridized onto the telomeric regions of all but three chromosomes (Chr. 11, 19, and 22), the centromeric regions of all but one chromosome (Chr. 8), and some interstitial chromosomal regions of chromosomes 3, 5, 8, and 11. **G.** Fosmid clone 180D9 with signals on the centromeric regions of chromosomes 1, 3, 5–9, 15, 19, 22–24, and Y. **H.** Fosmid clones 172B8, 180D9, and 171E1 hybridized collectively onto the metaphase of the white-cheeked gibbon. 172B8 was labeled by biotin-16-dUTP and visualized in blue; 180D9 was labeled by FITC-12-dUTP and visualized in green; 171E1 was labeled with SpectrumOrange-dUTP and visualized in red. The superposed region of simultaneous hybridization of 172B8 and 171E1 was visualized in purple; the superposed region of simultaneous hybridization of 180D9 and 171E1 was visualized in yellow. No chimeric clone was observed for all the fosmid clones tested.

different chromosomal distribution in different subgenera of gibbons, 172B8 will be useful for studying the heterochromatin evolution in gibbons, and 180D9 probably contains conservative centromeric repeats.

Fosmid clone specific for the Y chromosome

The BLAST search results showed that there was one clone (82H4) containing Y chromosome-specific sequence among the 100 randomly selected fosmid clones. Besides the human genome, we also did the sequence BLAST search between clone 82H4 and the genomes of macaque, cattle, cat, and mouse. The clone 82H4 blasted to the Y chromosomes of human and chimpanzee, but had hits on autosomes of macaque, cattle, cat, and mouse. Furthermore, we mapped it onto the metaphases of human, white-cheeked gibbon, chimpanzee, white-browed gibbon,

macaque, slow loris, cattle, and mouse. Hybridization signals were found on the Y chromosomes of human, white-cheeked gibbon, chimpanzee, and white-browed gibbon, but not in macaque, slow loris, cattle, cat, and mouse. The sequence of clone 82H4 is thus specific for the higher primates (apes).

Conclusion

In summary, we have constructed a white-cheeked gibbon fosmid library containing 192,000 clones. Based on the estimated insert size, the number of clones, and the putative genome size, this library should represent at least 2.5-fold coverage of the white-cheeked gibbon genome. We have demonstrated that this library has no obvious bias in chromosome coverage (chromosomal distribution) and no spurious chimeric clones in 100 randomly tested clones. As a preliminary effort, we have generated 196 fosmid end sequences and

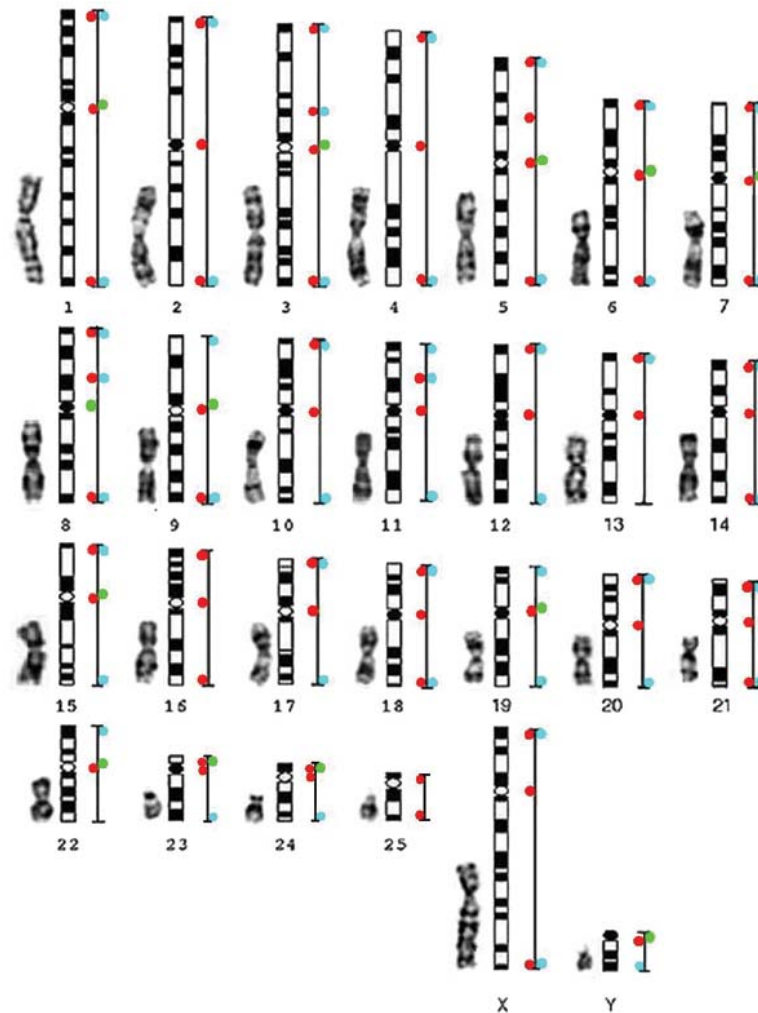


Fig. 4 Summary of the hybridization patterns of fosmid clones 172B8, 180D9, and 171E1 onto male white-cheeked gibbon chromosomes. The blue, green, and red dots denote hybridization signals of 172B8, 180D9, and 171E1, respectively.

mapped chromosome location for 93 fosmid clones of the white-cheeked gibbon. Future large-scale end sequencing and FISH mapping of this library will provide a useful resource for evolutionary synteny breakpoint studies as well as for determining the lineage-specific copy number variations and subtle inversions between humans and apes.

Materials and Methods

Construction and characterization of the fosmid library

The lymphoblast cell line (KCB 99002) of a male Yunnan white-cheeked gibbon with a normal karyotype ($2n=52$) was obtained from Kunming Cell Bank. High molecular weight genomic DNA was extracted from this cell line using the GeneElute mammalian ge-

nomeric DNA kit (Sigma, St Louis, USA). The size of this genomic DNA ranged from 25 to 148 kb, and mostly focused on 30 to 60 kb, thus, additional shearing was not necessary. This genomic DNA was directly end-repaired and then the size was selected as 33–48 kb fragments using FIGE. Ligation of the collected fragments into the fosmid vector pCC1FOS was performed according to the manufacturer's protocols (Copy control fosmid production kit; Epicentre Technologies, Madison, USA). The well-separated clones were picked and inoculated in Luria broth supplemented with 10% (v/v) glycerol in 384-well microtitre plates. After picking, the plates were incubated overnight at 37°C and then stored at -80°C. To evaluate the average insert size of clones, 146 clones were randomly selected from the white-cheeked gibbon fosmid library. Each fosmid clone DNA was extracted and completely digested using Not I, and then

the insert size was estimated by PFGE. The molecular weights of the fosmid inserts were calculated using the image analysis program Kodak 1D 3.5 based on the low-range size marker (New England BioLabs, Ipswich, USA) or fosmid control DNA (36 kb).

Fosmid end sequencing

One hundred fosmid clones were randomly picked for two-end sequencing. T7 primer (5'-TAATACGACTC ACTATAGGG-3') was used for 5' end sequencing, and pCC1TM/pEpiFOSTM RP-2 primer (5'-TACGC CAAGCTATTTAGGTGAGA-3') was used for 3' end sequencing. Sequences were analyzed with Lasergen software (Version 5.0; DNASTAR, Madison, USA). After trimming off the vector sequences, the sequences of insert DNA were blasted against the human genome sequences (<http://www.ncbi.nlm.nih.gov> or <http://genome.ucsc.edu>).

Cell culture, chromosomal preparation, and G-banding

The lymphoblast cell line of the white-cheeked gibbon was immortalized with Epstein-Barr virus and grown at 37°C in RPMI 1640 medium enriched with 15% newborn bovine serum. Before harvesting for chromosome analysis, the cell culture was treated with 0.03–0.05 µg/mL colchicine (Sigma) for 40 min. Chromosome preparations were made following standard procedures as previously described (33). G-banding before *in situ* hybridization was based on the classical trypsin/Giemsa staining procedure (34). Banded metaphases were captured with a Cohu CCD camera and analyzed with the Genus system (Applied Imaging, Grand Rapids, USA).

Single-color FISH with fosmid clones

The 100 fosmid clones used in end sequencing were mapped onto the chromosomes using FISH. All fosmid clone probes were labeled with biotin-14-dCTP by nick translation and hybridized to the prepared chromosomes, which followed the procedure described previously (35).

Multi-color FISH with fosmid clones

The three special fosmid clone probes that hybridized to telomere or centromere regions were labeled with biotin-16-dCTP for 172B8, FITC-12-dUTP for 180D9, and SpectrumOrange-dUTP for

171E1, respectively. The reaction system was the same as the single-color FISH reaction system, except that 0.5 µL ACG was used instead of 1.0 µL ATG, and biotin-16-dCTP or FITC-12-dUTP or SpectrumOrange-dUTP was used instead of biotin-14-dCTP. For the signal detection, biotin-labeled probes were detected with a layer of Cy5-avidin (1:500 dilution), and FITC-labeled probes were detected with a layer of rabbit-anti-FITC (1:250 dilution) followed by a layer of goat-anti-rabbit-FITC antibodies (1:125 dilution). After detection, slides were mounted in anti-fade AF1 solution (Citifluor Ltd., Canterbury, UK) containing 0.08 mg/mL DAPI (4'6-diamidino-2-phenylindole) and covered with 22×32 mm² coverslips.

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Authors' contributions

LC performed the experiment, conducted data analyses, and prepared the manuscript. JY, YL, JW, and WS joined LC in the experiment and data analyses. FY and WN supervised the project and co-wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

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