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Nucleotide sequence comparison of a chromosome rearrangement on human chromosome 12 and the corresponding ape chromosomes

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Abstract. Chromosome rearrangement has been considered to be important in the evolutionary process. Here, we demonstrate the evolutionary relationship of the rearranged human chromosome 12 and the corresponding chromosome XII in apes (chimpanzee, bonobo, gorilla, orangutan, and gibbon) by examining PCR products derived from the breakpoints of inversions and by conducting shotgun sequencing of a gorilla fosmid clone containing the breakpoint and a "duplicated segment" (duplicon). We confirmed that a pair of 23-kb duplicons flank the breakpoints of inversions on the long and short arms of chimpanzee chromosome XII. Although only the 23-kb duplicon on the long arm of chimpanzee chromosome XII and its telomeric flanking sequence are found to be conserved among the hominoids (human, great apes, and gibbons), the duplicon on the short arm of chimpanzee chromosome XII is suggested to be the result of a duplication from that on the long arm. Furthermore, the shotgun sequencing of a gorilla fosmid indicated that the breakpoint on the long arm of the gorilla is located at a different position 1.9 kb from that of chimpanzee. The region is flanked by a sequence homologous to that of human chromosome 6q22. Our findings and sequence analysis suggest a close relationship between segmental duplication and chromosome rearrangement (or breakpoint of inversion) in Hominoidea. The role of the chromosome rearrangement in speciation is also discussed based on our new results.

Human, chimpanzee, bonobo, gorilla, orangutan and gibbon are extant representative species of superfamily Hominoidea. The karyotypes of human and great apes (gibbons, or lesser apes, are not included in great apes) are well conserved, with a few apparent, large-scale chromosomal changes among these species, despite the considerable phenotypic differences between humans and the great apes (King and Wilson, 1975; Yunis et al., 1980; Yunis and Prakash, 1982). Primary examination through the use of G-banding cytogenetic techniques showed that the chromosomal differences separating the human and chimpanzee genomes consist of only ten euchromatic rearrangements: a telomere fusion between chimpanzee chromosome 12 and 13, resulting in human chromosome 2, and nine pericentric inversions (in human, chromosomes 1, 4, 5, 9, 12, 15, 16, 17, and 18; Yunis et al., 1980; Yunis and Prakash, 1982). Based on such close similarities, the Roman nomenclature was proposed to designate ape chromosomes based on homologies with the human karyotype (Creau-Goldberg et al., 1987). We follow this Roman nomenclature of ape chromosomes in this paper. Various breakpoints of chromosome rearrangements in human and apes have been discovered in recent

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studies (Nickerson and Nelson, 1998; Stankiewicz et al., 2001; Kehrer-Sawatzki et al., 2002; Eder et al., 2003; Locke et al., 2003).

Yunis and Prakash (1982) demonstrated through G-banding techniques that the chromosome XII of chimpanzee and gorilla experienced different inversions on the same chromosome location. Nickerson and Nelson (1998) showed that the two megaYACs from human 12p that span a pericentric inversion breakpoint in chimpanzee are also in a similar rearrangement in gorilla while the 12q15 YAC containing the inversion in chimpanzee was not found on chromosome XIIq of gorilla. The BAC clone sequences containing inversion breakpoints in chimpanzee chromosome XIIp and XIIq were described by Muzny et al. (1999a, b). These genomic sequences show that duplicated fragments (duplicons) are adjacent to the breakpoints of pericentric inversions on chimpanzee chromosome XII, while the duplicon is not repeated in homologous human chromosome 12 (D.L. Nelson, personal communication). Since information regarding breakpoints of gorilla compared to chimpanzee is limited, a more detailed analysis is required to delineate the difference in the inversion events between these two species.

There are segmental duplication patterns in the genome that are module-like in structure and organized in a patchwork fashion in which different modules are concatenated to form larger complex arrays (Eichler, 2001). These duplicons may be associated with chromosome rearrangements between species; for example, a sequence comparison between human chromosome 19 and the corresponding mouse chromosomes reveal regional gene family duplications at ten out of fifteen chromosome breakpoints (Dehal et al., 2001). Such chromosome structure fluidity could provide the underlying mechanism for the construction of speciation barriers by creating regions with an inherent proclivity to rearrange (Eichler, 2001).

In this study, we examined the homology of published chimpanzee BAC clone sequences containing breakpoints of both inversions and duplications, through (1) PCR-based sequencing of these BAC clones to identify homology among Hominoidea for local sites and (2) through homology searches of these BAC clone sequences. We also examined the difference in XIIq breakpoints between chimpanzee and gorilla by shotgun sequencing of a gorilla fosmid clone obtained by Kim et al. (2003).

Materials and methods

Polymerase Chain Reaction (PCR) of breakpoints and DNA sequencing

PCRs were performed under the following conditions: each 20-μl reaction contained 1–5 ng template DNA, 1× EX *Taq* amplification buffer, 0.5 units EX *Taq* enzyme (TaKaRa), 0.5 mM each primer, and 200 μM each dNTP. The DNA was initially denatured at 94 °C for 5 min, then amplified for 40 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, and finally extended at 72 °C for 3 min. PCR primer pairs were designed to amplify a sequence spanning the breakpoint of chimpanzee BAC clone sequence and the corresponding human BAC clone sequence (Fig. 1). Using these primers, human *(Homo sapiens)*, chimpanzee *(Pan troglodytes)*, bonobo *(Pan paniscus)*, gorilla *(Gorilla gorilla)*, orangutan *(Pongo pygmaeus)*, and two gibbon species *(Hylobates agilis* and *Hylobates lar)* genomic DNA extracted from blood was amplified by PCR. PCR products were checked by electrophoresis on a 1.5–1.8% agarose gel (100 volts constant, 30–40 min).

Shotgun sequencing of a gorilla fosmid clone

A gorilla fosmid clone library stored in −80 °C in glycerol medium at the National Institute of Genetics (constructed by Kim et al., 2003) was screened by PCR using primer pairs designed against a human BAC clone sequence. The PCR conditions and electrophoresis are described above. A positive clone was cultured overnight and DNA was extracted from an *E. coli* cell using a Qiagen Large kit. The extracted DNA was sheared using HydroShear (Genomic Instrumentation Services) using the following conditions: volume 100 ml, cycle 20, speed 5. The sheared DNA was separated on 1.0% Seakem GTG agarose, Tm = 92 °C, and a 500-bp to 3-kb fragment of DNA was excised and purified using a Qiagen kit. The purified DNA was treated to produce blunt-ends using a TAKARA DNA blunting kit, followed by ligation into a puc118/*Hin*cII BAP vector, and the ligated DNA/plasmid was transformed into TOYOBO competent high DH5α cells. Transformed bacteria were spread on LB/Amp/X-Gal plates and a positive clone was selected for sequencing on an ABI 9600 automated sequencer. The obtained sequences were base-called using Phred (Ewing et al., 1998; Ewing and Green, 1998), assembled with Phrap, masked vector sequenced by Cross Match, and subsequently edited using Consed (Gordon et al., 1998, 2001).

BLAST searches

The obtained fosmid clone contig sequences were used in a BLAST search for corresponding sequences in the DDBJ/EMBL/GenBank International Nucleotide Sequence Database and aligned by the BLAST program (Altschul et al., 1997) of the DDBJ web service (www.ddbj.nig.ac.jp).

Calculation of evolutionary distances

Repeat Masker at the "Repeatmasker Web Server" (Smit and Green, 2003) was used on the common homologous region in three sequences of AC005294 on human 12q15, AC007214 on chimpanzee XIIq, and the obtained gorilla fosmid sequence to mask repetitive sequences. Alignments were produced using Clustal W (Thompson et al., 1994) of the DDBJ web site (www.ddbj.nig.ac.jp). Multiple-alignment data was analyzed using DAMBE ver. 4.2.7 (Xia and Xie, 2001) and *K* values were calculated using both the Kimura (1980) two-parameter model and the model of Tamura and Nei (1993).

Results

Correspondence of the published chimpanzee BAC clone sequence to its human counterpart

BLAST homology searches were conducted to assure the correspondence of published chimpanzee BAC clone sequence to the human counterpart, including their breakpoints. Figure 1 depicts the correspondence of two published chimpanzee BAC clone sequences (XIIp; AC006582, XIIq; AC007214) to their human counterparts (12p12; AC011604, 12q15; AC005294) based on BLAST searches. Half of the human BAC clone (AC011604) located at 12p12 (Hp1–6 in Fig. 1) corresponds to chimpanzee BAC clone sequence (AC006582, Cp10–Cp5 in Fig. 1), while the other half of this chimpanzee sequence (Cp5–Cp2 in Fig. 1) corresponds to the human BAC clone (AC005294) located at 12q15 (Hq4–Hq1 in Fig. 1). A part of this human region (Hq3–Hq6 in Fig. 1) also has a corresponding region in chimpanzee BAC clone AC007214 (Cq4–Cq2 in Fig. 1). Consequently, a 23-kb region in human (shown in a checkered pattern, Hq3–4 in Fig. 1) corresponds to two regions in chimpanzee XIIp and XIIq. Based on this result, we defined the breakpoint on human 12q15 (Hj) and the chimpanzee chromosome breakpoint (Cj), as shown in Fig. 1.

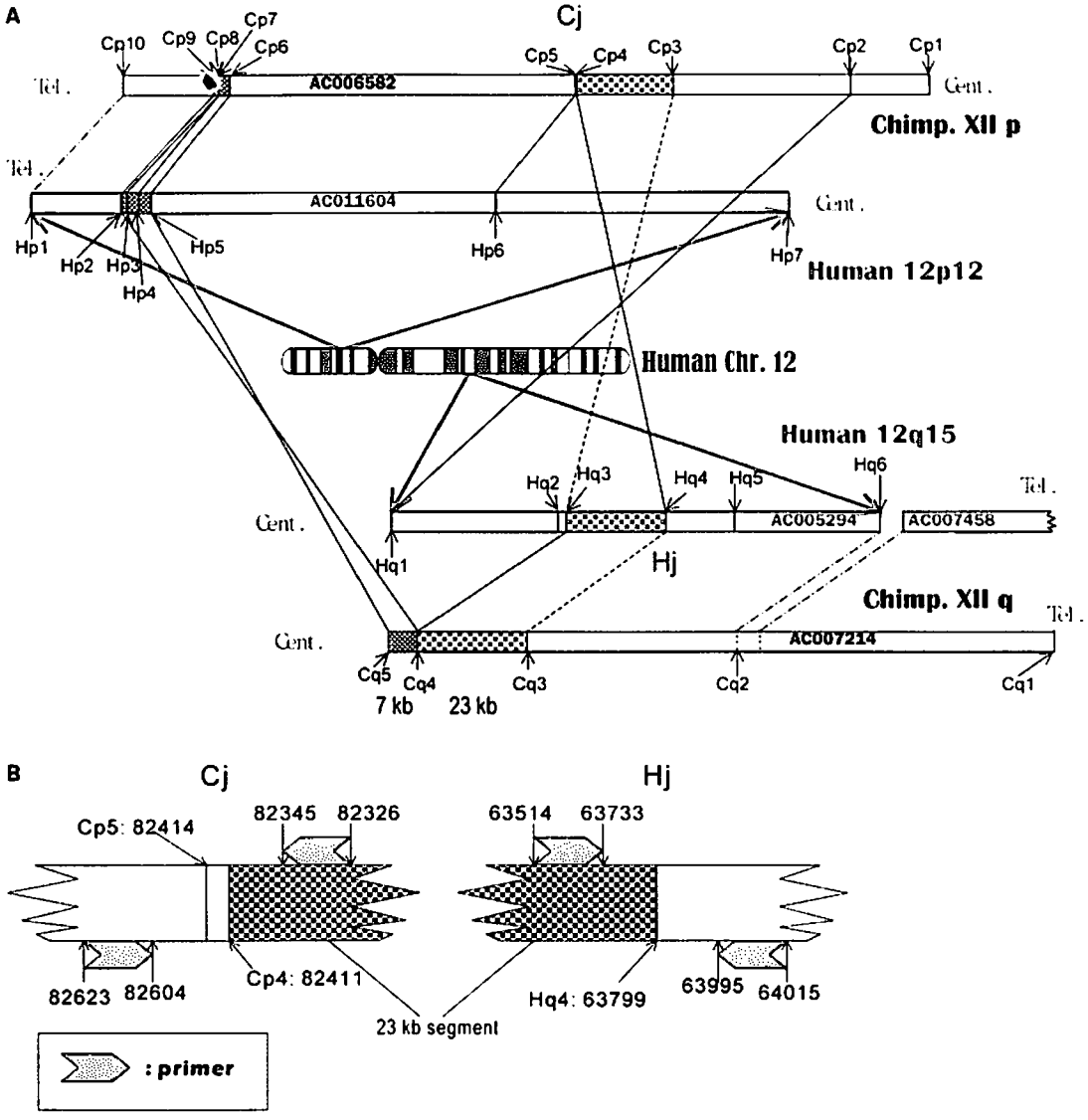

Fig. 1. Correspondence of the published chimpanzee BAC clone sequence to the human counterpart. **(A)** Thick arrows from human chromosome 12 indicate the position of human BAC clone sequences (counterpart) in human chromosome 12 (i.e., 12p12 and 12q15). Sequences corresponding to the human BAC clone sequences are depicted with thin lines. Two duplicated regions found by D.L. Nelson and his colleagues (personal communication) are depicted with larger ("23-kb duplicon") and smaller ("7-kb duplicon") checkered pattern. Nucleotide positions in each entry are depicted as follows, Cp1: bp1, Cp2: bp18480, Cp3: 59273, Cp4: 82411, Cp5: 82414, Cp6: 161506, Cp7: 164445, Cp8: 164452, Cp9: 165259, Cp10: 186092, Hp1: 1, Hp2: 20828, Hp3: 21635, Hp4: 24575, Hp5: 27545, Hp6: 107108, Hp7: 175606, Hq1: 1, Hq2: 38746, Hq3: 40621, Hq4: 63799, Hq5: 77985, Hq6: 113762, Cq1: 1, Cq2: 74534, Cq3: 124431, Cq4: 147956, Cq5: 154685. PCR primer pairs are designed on the position indicated by "Cj" and "Hj". **(B)** Magnification of Cj and Hj sites. Primer positions used for PCR and sequencing are indicated by a block arrow. Numbers show the nucleotide position in AC006582 and AC005294, respectively.

PCR of breakpoints and DNA sequencing

Figure 2 shows the results of PCR using primers designed to span the breakpoint on human 12q15 (Hj) and the breakpoint on chimpanzee chromosome XIIp (Cj). In the Hj site, PCR of the genomic DNA of all examined species amplified products whose lengths are equal to that between the primers on the published sequence (AC005294, Fig. 1B). Sequencing of these PCR products confirmed that they were the targeted amplicons. This suggests that the Hj site has a conserved chromosome location across all Hominoidea. On the contrary, the PCR product of the size expected in the Cj site was found only in chimpanzee and bonobo. About 1 kb length of untargeted PCR products were observed in human, gorilla, orangutan and bonobo (Fig. 2B). Each PCR product of the Cj site was sequenced and a BLAST search was conducted (Table 1). The approximate 1-kb PCR products (human 991 bp, bonobo 1,204 bp, gorilla 1,108 bp in Table 1) and approximate 300-bp PCR products (gorilla 335 and 262 bp, gibbon 299 bp in Table 1) were not homologous to the chimpanzee short arm (XIIp). The fact that the Hj site is found in all Hominoidea, but the Cj site is found

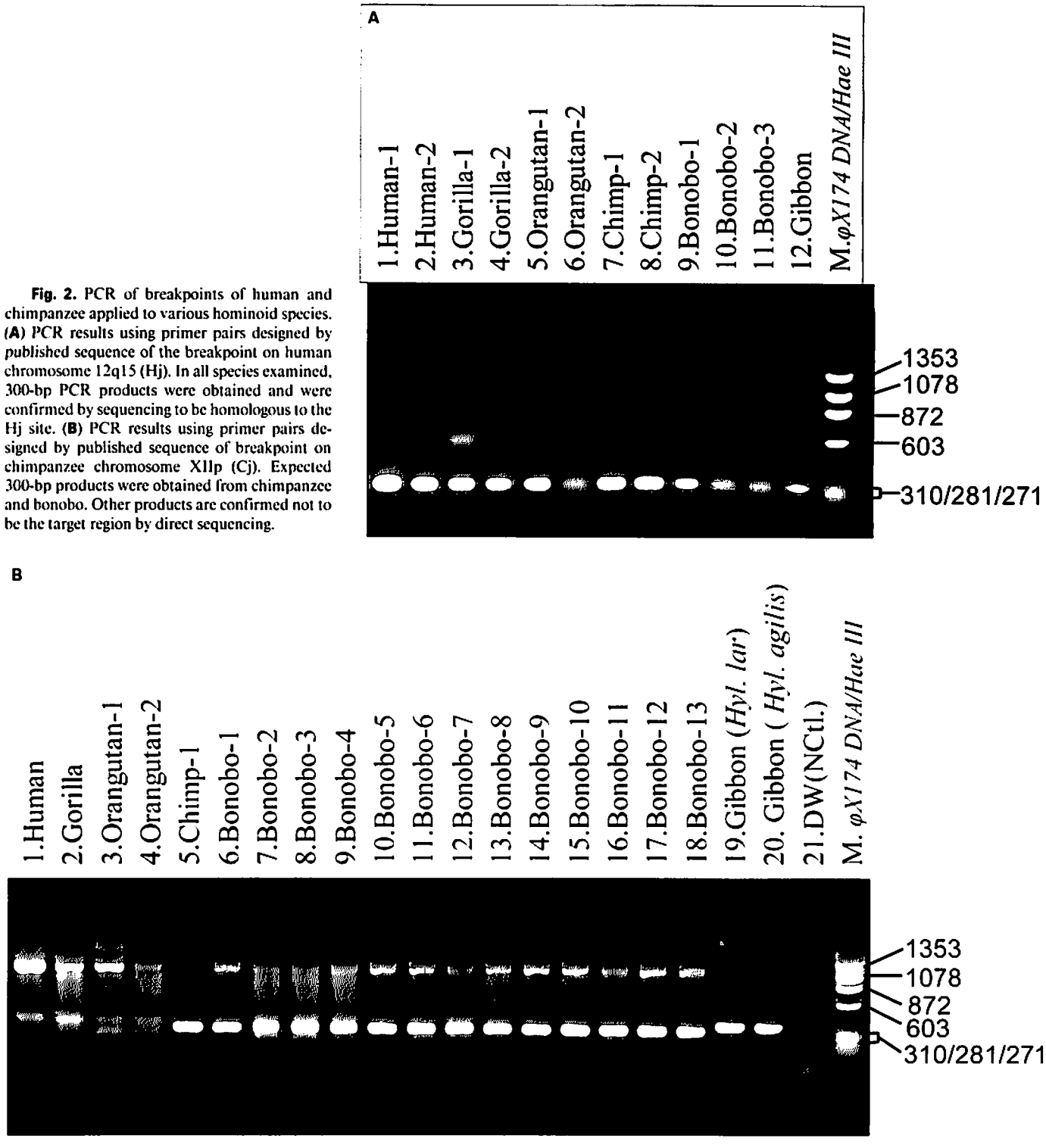

Fig. 2. PCR of breakpoints of human and chimpanzee applied to various hominoid species. **(A)** PCR results using primer pairs designed by published sequence of the breakpoint on human chromosome 12q15 (Hj). In all species examined, 300-bp PCR products were obtained and were confirmed by sequencing to be homologous to the Hj site. **(B)** PCR results using primer pairs designed by published sequence of breakpoint on chimpanzee chromosome XIIp (Cj). Expected 300-bp products were obtained from chimpanzee and bonobo. Other products are confirmed not to be the target region by direct sequencing.

only in the *Pan* lineage, suggests divergent evolution in the flanking regions of these breakpoint sites. Furthermore, it also suggests that in chimpanzee the duplicon flanked by the Hj site (duplicon on XIIq) is the original sequence while the duplicon flanked by the Cj site (duplicon on XIIp) is the result of a duplication from the original one.

Figure 3 shows multiple alignments of the sequences of the Hj and Cj sites amplified by the primers shown in Fig. 1B. These sequence data were deposited to the DDBJ/EMBL/GenBank International Nucleotide Sequence Database under accession numbers AB181930–AB181942. The two published sequences used for designing PCR primers (AC005294 and

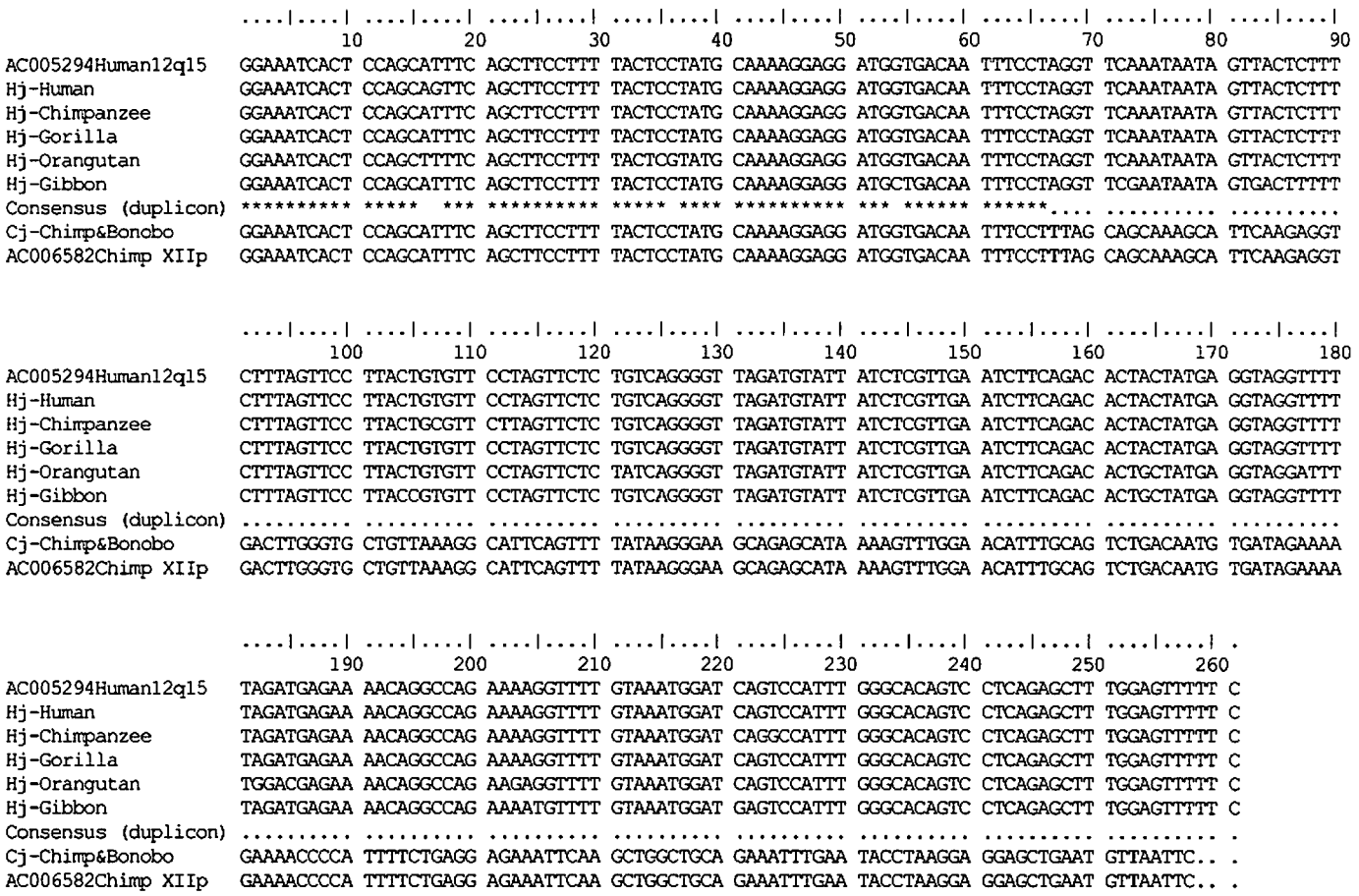

Fig. 3. Multiple alignment of sequences generated from the PCR products at Hj and Cj sites with published sequences. Hj designates the PCR product sequence at the breakpoint of human 12q, and Cj designates that of chimpanzee XIIp.

Table 1. Results of BLAST searches for various PCR product sequences

Species	PCR product length	Entry of homologous region	Start	End	Identity
Human	991 bp	AC133606 (*Homo sapiens* BAC clone RP11-494P20 from chromosome 2)	1931 bp	2753 bp	822/823 (99 %)
Bonobo	1204 bp	AC133606 (*Homo sapiens* BAC clone RP11-494P20 from chromosome 2)	1931 bp	2981 bp	1036/1051 (98 %)
Bonobo	297 bp	AC006582 (*Pan troglodytes* 12cp12 BAC RPCI43-77C18)	82327 bp	82623 bp	296/297 (99 %)
Gorilla	1108 bp	AC133606 (*Homo sapiens* BAC clone RP11-494P20 from chromosome 2)	1931 bp	2983 bp	1040/1053 (98 %)
Gorilla	335 bp	AL441923 (*Homo sapiens* from clone RP11-104D21 on chromosome Xp11.21-11.23)	33010 bp	33333 bp	316/324 (97 %)
Gorilla	262 bp	AC093796 (*Homo sapiens* BAC clone RP11-301H24 from chromosome 4)	55913 bp	56141 bp	226/229 (98 %)
Gorilla	258 bp	AC022778 (*Homo sapiens* clone RP11-172E10 (or AC012533 clone RP11-62E9) on chromosome 8q21.13)	109582 bp (189559 bp)	109830 bp (189807 bp)	246/249 (98 %)
Gibbon	299 bp	AC093537 (*Homo sapiens* clone RP11-8L21 (AC093531 clone RP11-405L7) on chromosome 5)	2420 bp (31613 bp)	2718 bp (31315 bp)	289/299 (96 %)

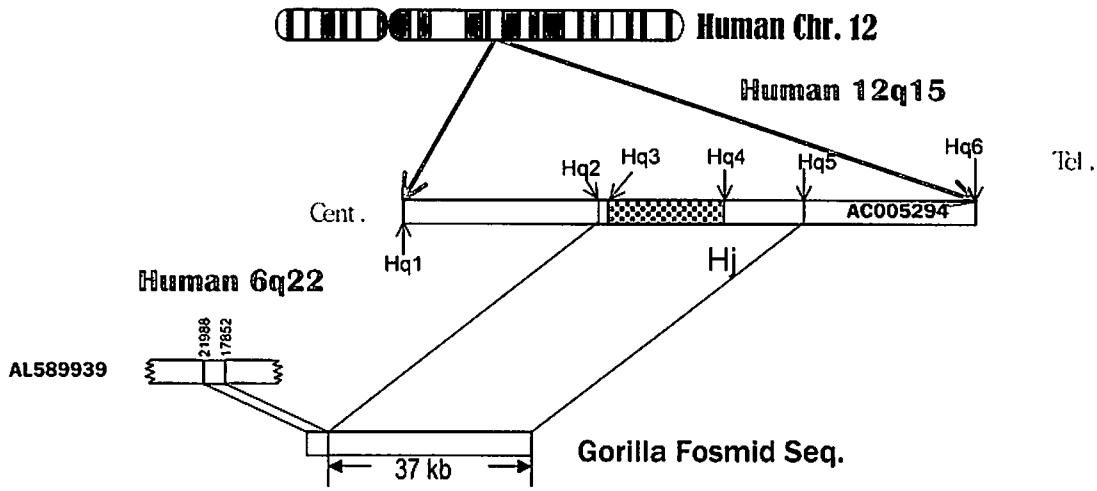

Fig. 4. Correspondence of the assembled gorilla fosmid clone sequence to its human counterpart. As shown in Fig. 1, thick arrows from human chromosome 12 indicate the position of human BAC clone sequences in human chromosome 12 (i.e., 12q15). Sequences corresponding to the human BAC clone sequences are depicted with thin lines. Numbers on sequences represent the nucleotide position in each entry. The duplicated region found by D.L. Nelson and his colleagues (personal communication) is depicted with checkered pattern. PCR primer pairs are designed on the position indicated by "Hj". Our assembled gorilla fosmid sequence showed correspondences to human 12q15 and 6q22.

AC006582) are juxtaposed. The published human BAC sequence, AC005294, is shown from 63,734 to 63,994 nt, while that of chimpanzee BAC sequence AC006582 is shown from 82,346 to 82,603 nt. Because the Cj site sequences of chimpanzee and bonobo were identical, "Cj-chimp&bonobo" represent both sequences. All the amplification products show homology with each other at nucleotides 1–66, which corresponds to the sequence within the "23-kb duplicon". The 66th nucleotide corresponds to Hq4 in the Hj site and Cp4 in the Cj site, respectively, in Fig. 1B.

Shotgun sequencing of the gorilla fosmid clone

PCR screening of 147,456 gorilla fosmid clones detected two positive clones containing the Hj site (GGFP-343M12 and GGFP-225L7). One such clone (GGFP-343M12) containing the Hj site was chosen for sequence analysis. The homologous position in chimpanzee XIIq was adjacent to the duplicon and, therefore, considered to be important for duplication and chromosomal inversion breakpoints on the chimpanzee chromosome XII. The shotgun sequence reaction was conducted bidirectionally using both ends on 10 of the 384 well plates (i.e., 7,680 reactions). The number of shotgun sequences used for the final contiguous sequence construction was approximately 4,500, and the total length of the assembled sequence, containing three gaps, was 43 kb. This sequence corresponds to human 12q15 (Hq2 to Hq5 on Figs. 1 and 4) containing a sequence homologous to the duplicated region (Hq3 to Hq4 on Figs. 1 and 4) and to the breakpoint on chimpanzee chromosome XIIq (Hq3 corresponding to Cq4 on Figs. 1 and 4). This result indicates that the breakpoint of the chromosome inversion on XIIq in gorilla (Hq2 on Figs. 1 and 4) is different from that of chimpanzee (Hq3 or Cq4 on Figs. 1 and 4). Since the human 12q15 region from Hq3 to Hq5 is homologous to chimpanzee XIIq and the gorilla fosmid sequence, this also confirms that the duplicon on chimpanzee XIIq is ancestral to that on XIIp. Two of the three gap sites consist of single nucleotide repeats (i.e., poly-A). BLAST search for the obtained gorilla fosmid sequence in the human genome database showed that the gorilla fosmid sequence consists of a region homologous to human 12q15 and a region homologous to 6q22 (Fig. 4). The third gap in the gorilla fosmid sequence corresponds to a 294-bp segment in the human sequence. The Repeat Masker (Smit and Green, 2003) indicated that 281 of the 294 bp in the third gap are an *Alu* insertion. The fosmid clone sequence data was deposited to the DDBJ/EMBL/GenBank International Nucleotide Sequence Database under accession numbers AB181928 and AB181929.

Discussion

Duplication in chimpanzee chromosome XII

The results of PCR on the Hj and Cj sites indicate that the duplicon of chimpanzee XIIq is the ancestral type of the duplication found in Hominoidea as it is present in all of Hominoidea, including *Pan*, whereas the Cj site is present only in *Pan*. The fosmid clone sequencing on gorilla confirmed that the homologous region of the gorilla also contains the sequence of the 23 kb duplicon of the chimpanzee.

Breakpoint of gorilla XIIq

Hq3 on human 12q15 is a breakpoint in chimpanzee, but not in the inversion in gorilla (Fig. 4). This confirmed that the inversion events of chromosome XII in chimpanzee and gorilla

Table 2. Number of nucleotide substitutions per 100 sites *(K)* in the translocational region of the human chromosome 12 and corresponding sequence of chimpanzee and gorilla

	Human	Chimpanzee
Chimpanzee	1.44 (0.08)	
Gorilla	1.88 (0.09)	1.90 (0.09)

Repetitive sequences are excluded from this analysis. Both the Kimura (1980) two-parameter distance and the Tamura and Nei (1993) distance showed almost equal values in each pair. The values in parenthesis are standard deviation in Kimura's two-parameter (1980) distance.

are different. The breakpoint of gorilla was detected 1.9 kb centromeric from that of the chimpanzee XIIq breakpoint. These sites correspond to Hq2 and Hq3 on human 12q15, respectively. The gorilla's breakpoint flanks the region homologous to human 6q22, which is 4 kb from the end of the fosmid sequence (Fig. 4). According to a homology search of the human genome sequence, there is no evidence of duplication events between human chromosomes 12 and 6q22 (Fig. 4). It may be necessary to exclude the possibility of chimerization in the fosmid clone in a future study.

The finding that an independent inversion occurred proximal to a duplicon in the chimpanzee may support the importance of duplication in chromosome rearrangement. Eichler (2001) pointed out an association between duplicated regions and sites of chromosome rearrangement between species. Both chromosome-specific and transchromosomal duplications increase the likelihood of secondary rearrangements leading to additional inversions, deletions and duplications (Eichler, 2001). The region examined here may be an example of these phenomena.

Chromosomal rearrangements and speciation

A new class of chromosomal speciation models suggests that chromosomal changes are strong barriers because they reduce recombination in heterokaryotypes (Noor et al., 2001; Rieseberg, 2001; Ortiz-Barrientos et al., 2002; Navarro and Barton, 2003a). Such strong barriers would facilitate divergence in the rearranged region during the time when the diverging populations have limited gene flow. Navarro and Barton (2003b) analyzed two data sets (Ebersberger et al., 2002; Yi et al., 2002) and showed that nucleotide substitution was slightly higher in rearranged chromosomes.

Since chromosome 12 (XII) is rearranged (it has an inversion) the nucleotide substitution rate is expected to be higher than in other, collinear chromosomes. We thus estimated the nucleotide divergence of the rearranged sequences of human, chimpanzee, and gorilla. Table 2 shows the number of nucleotide substitutions per 100 sites *(K)* in the non-repetitive region of chromosome 12 (XII) of human, chimpanzee, and gorilla. Distances between human and gorilla and that between gorilla and chimpanzee are very similar (the amount of divergence was ca. 1.9%), while the value between chimpanzee and human is slightly larger than the genomic average of ca. 1.2% (Fujiyama et al., 2002).

Navarro and Barton (2003b) calculated the K values between human and chimpanzee based on two data sets: (1) 447,330 bp of non-coding, non-repetitive sequence from different chromosomes by Yi et al. (2002) and (2) 8,859 sequence pairs encompassing 1.9 Mb of genomic sequence by Ebersberger et al. (2002). They then compared the K values between rearranged and colinear chromosomes. The K values calculated using the results of Yi et al. (2002) showed significant differences between rearranged and collinear chromosomes (1.40 versus 1.15%), while those calculated using the data of Ebersberger et al. (2002) did not differ significantly (1.25 versus 1.23%). Navarro and Barton (2003b) explained that the measures are as expected if gene flow is lower in rearranged chromosomes. They attributed the higher divergence in rearranged chromosome to an accumulation of favored alleles in the rearranged region. In the 37 kb of the homologous region of the human 12q15 (Hq3–Hq5 on Figs. 1 and 4), the K value between human and chimpanzee was 1.44, which is slightly higher than the average of those on rearranged chromosomes studied by Yi et al. (2002). Thus, our K value is consistent with the new model of speciation. However, this new model of chromosome speciation was recently questioned (Lu et al., 2003; Hey, 2004), so it is possible that our result on nucleotide divergence can be explained by another model of evolution.

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