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The chimpanzee-specific pericentric inversions that distinguish humans and chimpanzees have identical breakpoints in *Pan troglodytes* and *Pan paniscus* ☆

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Abstract

Seven of nine pericentric inversions that distinguish human (*HSA*) and chimpanzee karyotypes are chimpanzee-specific. In this study we investigated whether the two extant chimpanzee species, *Pan troglodytes* (common chimpanzee) and *Pan paniscus* (bonobo), share exactly the same pericentric inversions. The methods applied were FISH with breakpoint-spanning BAC/PAC clones and PCR analyses of the breakpoint junction sequences. Our findings for the homologues to *HSA* 4, 5, 9, 12, 16, and 17 confirm for the first time at the sequence level that these pericentric inversions have identical breakpoints in the common chimpanzee and the bonobo. Therefore, these inversions predate the separation of the two chimpanzee species 0.86–2 Mya. Further, the inversions distinguishing human and chimpanzee karyotypes may be regarded as early acquisitions, such that they are likely to have been present at the time of human/chimpanzee divergence. According to the chromosomal speciation theory the inversions themselves could have promoted human speciation.
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Keywords: Pericentric inversion breakpoints; Human–chimpanzee comparisons; Chromosome evolution; FISH; Breakpoint-junction PCR

The chimpanzee and human lineages diverged from each other about 5–6 Mya [1]. The chimpanzee lineage is now represented by two extant species: *Pan troglodytes* (*PTR*; common chimpanzee) and *Pan paniscus* (*PPA*; bonobo or pygmy chimpanzee). These two chimpanzee species live in different geographical areas separated by the Congo River and manifest distinct behavioral, morphological, and genetic traits [2]. It is estimated that these two species became separated 0.86–2 Mya [3,4].

Although the karyotypes of the common chimpanzee and bonobo are very similar, a few differences have been noted [5]. With the use of chromosome banding techniques a *PPA*-specific pericentric inversion of the chromosome homologous to *HSA* 2q and an insertion in the region equivalent to *HSA* 7q were identified. Further, a large heterochromatin insertion is also present on the *PTR* homolog of *HSA* 13q, which is absent in human and bonobo. Finally, the chromosome homologous to *HSA* 22 is acrocentric in *PTR*, whereas it is metacentric in *PPA*, owing to the addition of heterochromatin.

In contrast to the strong karyotypic similarity manifested by the common chimpanzee and bonobo, the human and chimpanzee karyotypes are distinguished by as many as nine pericentric inversions, the fusion that gave rise to *HSA* 2, as well as marked variation in the amount of constitutive heterochromatin [6]. The nine pericentric inversions that distinguish the chromosomes of the human and common chimpanzee involve the homologues of *HSA* 1, 4, 5, 9, 12, 15,

Abbreviations: BAC, F-factor-based bacterial artificial chromosome; *HSA, Homo sapiens*; PAC, P1-derived artificial chromosome; *PPA, Pan paniscus* (bonobo); *PTR, Pan troglodytes* (common chimpanzee).

☆ Sequence data from this article have been deposited with the GenBank Data Library under Accession Nos. DQ000175–DQ000195 and DQ002477–DQ002479.

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16, 17, and 18. Comparative analysis, employing gorilla (*Gorilla gorilla*) and orangutan (*Pongo pygmaeus*) as outgroups, has revealed that the inversions of *HSA* 1 and *HSA* 18 occurred in the human lineage, whereas the other seven inversions are chimpanzee-specific [reviewed in [7]].

The inversions that distinguish humans and chimpanzees have attracted considerable interest as potential drivers of the process of speciation that split the human and chimpanzee lineages [8–15]. Therefore it is critically important to provide precise definitions of these inversions at the DNA sequence level.

In the present paper, we provide a definitive molecular comparison of the inversions present in the common chimpanzee and the bonobo. This study therefore extends the earlier work of Nickerson and Nelson [16], who refined the breakpoints of three of the chimpanzee-specific inversions to 1- to 2-Mb genomic intervals using YACs in bonobo and chimpanzee. Although the chimpanzee and the gorilla appear from examination of chromosome banding pattern to share the same pericentric inversion relative to *HSA* 12, molecular analyses demonstrated that the breakpoints were distinctly different in the two species [16,18]. In this study, fluorescence in situ hybridization (FISH) analyses have been performed with BAC or PAC probes identified during the previously reported characterization of the pericentric inversions that distinguish the human and common chimpanzee karyotypes [7,17–21]. With the exception of the characterization of the inversion of *PTR* 18 homologous to *HSA* 16 [20], in none of these previous studies has the bonobo been included. However, to estimate the evolutionary age of these inversions it is necessary to compare the exact inversion breakpoint regions in bonobo and the common chimpanzee. We have therefore analyzed these breakpoints in detail by PCR and sequencing of the respective inversion junction fragments. Our analyses involved six of the seven chimpanzee-specific inversions, the seventh (equivalent to *HSA* 15) having already been narrowed down to a 600-kb interval of the human genome consisting of entirely duplicated material [22]. Taken together, these studies indicate that at the DNA sequence level, the six inversions have identical breakpoints in the common chimpanzee and the bonobo.

Results

FISH with human breakpoint-spanning BACs/PACs

We initially performed FISH with human BAC/PAC clones that span the two inversion breakpoints of *PTR* chromosomes homologous to *HSA* chromosomes 4, 5, 9, 12, 16, and 17 as summarized in Table 1. For each probe, we observed FISH signals on *PPA* chromosomes that were split by the inversion (Fig. 1 and data not shown). This implies that the inversion breakpoints in the common chimpanzee as well as in the bonobo map to the same genomic regions.

FISH with breakpoint-spanning BACs from the common chimpanzee

To verify this conclusion, we performed additional FISH analyses with breakpoint-spanning BACs from the genome of the common chimpanzee. These BACs yielded single signals on *PTR* chromosomes but split signals on *HSA* chromosomes as previously determined. The results of these experiments are summarized in Table 2. With *PTR* BACs that detect the inversion breakpoints in *HSA* 4p, 4q, 5p, 5q, 9q, 17p, and 17q, we observed single signals in the orthologous regions of *PPA*. These findings can be taken as further confirmation that the breakpoints are identical in terms of their locations between *PPA* and *PTR*. In contrast to these inversion breakpoints, the breaks in the *PTR* chromosomes homologous to *HSA* 9p, 12p,

Table 1
Summary of FISH results with human breakpoint-spanning BAC/PACs

BAC/PAC acronym	Chromosomal location	Chromosomal coordinates (Mb)[a]	Localization in		
			HSA	*PTR*	*PPA*
RP11-779N22[b] (AC110811)	4p13	44,656,078–44,677,181	4p	p + q	p + q
RP11-8N8[b] (AC108021)	4q21.23	86,174,177–86,360,148	4q	p + q	p + q
RP11-35A11[c] (AC113389)	5p14.3–p15.1	18,434,022–18,604,727	5p	p + q	p + q
RP11-432G16[c] (AC104125)	5q15	95,843,232–96,023,456	5q	p + q	p + q
RP11-259A5[d] (BX664724)	9p12	42,421,610–42,550,187	9p	p + q	p + q
RP11-507D14[d] (AL137849)	9q21.33	86,028,571–86,160,254	9q	p + q	p + q
RP11-80N2[e] (AC011604)	12p12.2	20,833,482–21,009,087	12p	p + q	p + q
RP3-491B7[e] (AL606524)	12q15	66,593,873–66,695,640	12q	p + q	p + q
CTD-2144E22[f] (AC135776)	16p11.2	34,030,652–34,199,197	16p	p + q	p + q
RP11-696P19[f] (AC106819)	16q11.2	44,943,303–45,066,095	16q	p + q	p + q
RP1-179H24[g] (AF503578)[h]	17p13.1	7,868,375–7,878,854	17p	p + q	p + q
RP5-1029K10[g] (AC006487)	17q21.33	44,918,026–45,104,642	17q	p + q	p + q

[a] Ensembl Genome Browser, release 30.35c.
[b] Ref. [7].
[c] Ref. [21].
[d] Ref. [19].
[e] Ref. [18].
[f] Ref. [20].
[g] Ref. [17].
[h] The accession number refers to the sequenced breakpoint-spanning fragment of PAC RP1-179H24.

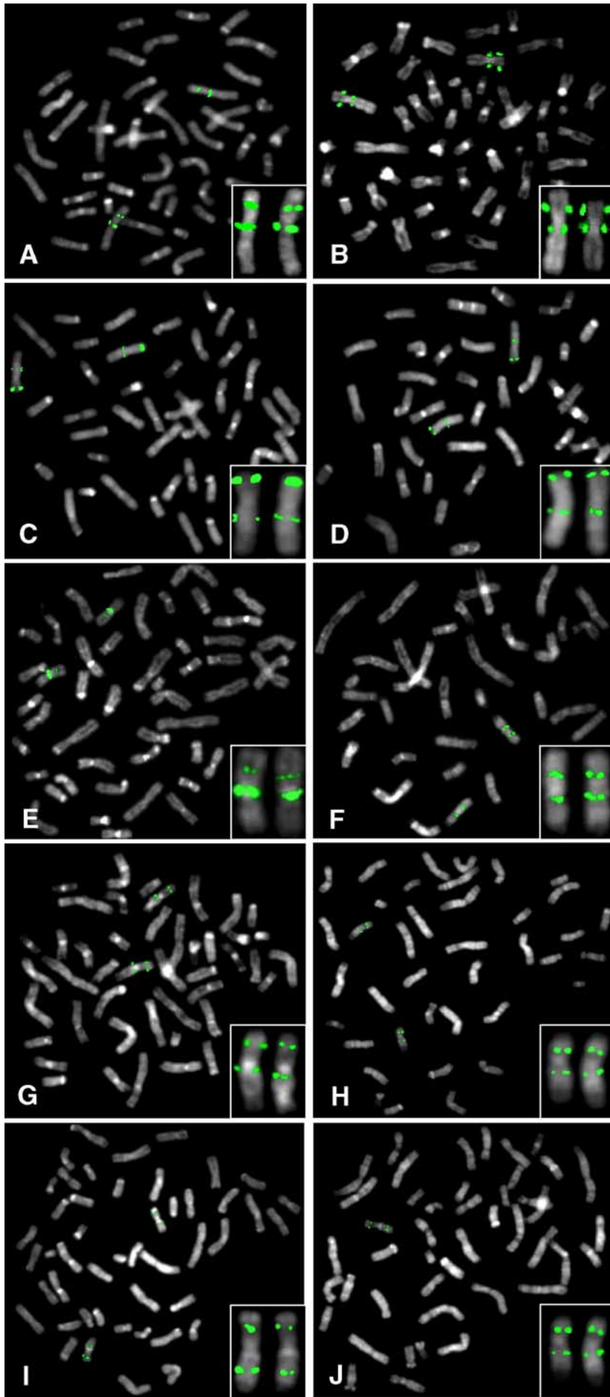

Fig. 1. Bonobo (*PPA*) metaphases after hybridization with human breakpoint-spanning BAC/PACs. (A) RP11-779N22 from *HSA* 4p, (B) RP11-8N8 from *HSA* 4q, (C) RP11-35A11 from *HSA* 5p, (D) RP11-432G16 from *HSA* 5q, (E) RP11-259A5 from *HSA* 9p, (F) RP11-507D14 from *HSA* 9q, (G) RP11-80N2 from *HSA* 12p, (H) RP3-491B7 from *HSA* 12q, (I) RP1-179H24 from *HSA* 17p, and (J) RP5-1029K10 from *HSA* 17q.

and 12q mapped to duplicated sequences [18,19]. Correspondingly, the chimpanzee BACs that span the breakpoints in regions homologous to *HSA* 9p, 12p, and 12q yielded signals on both the p- and the q-arm of the homologous chromosomes in *PPA* and *HSA* (data not shown). These findings indicate that in the bonobo, as well as in *PTR*, duplicated sequences were found within the orthologous breakpoint regions.

Table 2
Summary of FISH results with breakpoint-spanning BAC clones from the common chimpanzee (*PTR*) genome

Chromosomal position *PTR* (*HSA*)	Chimpanzee BACs	Accession No.[a]	FISH signals in		
			HSA	*PTR*	*PPA*
PTR 3p (*HSA* 4)	RP43-59P20	AY335550	p + q	p	p
PTR 3q (*HSA* 4)	RP43-41D24	AY335551	p + q	q	q
PTR 4p (*HSA* 5)	RP43-001C16	AG141159 AG141160	p + q	p	p
PTR 4q (*HSA* 5)	RP43-023F12	AG158191 AG158192	p + q	q	q
PTR 11p (*HSA* 9)	RP43-163C1	AY569337	p + q	p + q	p + q
PTR 11q (*HSA* 9)	RP43-156H15	CW691359 CW691360	p + q	q	q
PTR 10q (*HSA* 12)	RP43-77C18	AC006582	p + q	p + q	p + q
PTR 10q (*HSA* 12)	RP43-135M19	AC007214	p + q	p + q	p + q
PTR 18p (*HSA* 16)	RP43-007E19	AG145869 AG145870	p + q	p	p + q
PTR 18q (*HSA* 16)	RP43-001I03	AG141421 AG141422	p + q	q	p + q
PTR 19p (*HSA* 17)	RP43-141Q35	AF503579	p + q	p	p
PTR 19q (*HSA* 17)	RP43-134L13	AY117035	p + q	q	q

[a] The accession numbers refer to the sequenced breakpoint-spanning fragments or the end sequences of the respective BACs.

Finally, hybridization of common chimpanzee BACs (RP43-007E19 and RP43-001I03) spanning the inversion breakpoints of *PTR* 18 (homologous to *HSA* 16) yielded split signals on the homologous bonobo chromosomes that were indicative of a bonobo-specific duplication in addition to the pericentric inversion (Figs. 2 and 3).

PCR analysis of the breakpoints

To compare the breakpoints at the nucleotide sequence level, we performed PCR experiments that amplified DNA sequence across the inversion breakpoints. Primer design utilized *PTR* sequences that flank the respective breakpoints. The primers used for these analyses are listed in Table 3. We obtained PCR products of the expected lengths with genomic DNA from both *PTR* and *PPA*. However, this was not the case using human genomic DNA as a template. We next confirmed the authenticity of the respective PCR products by sequence analyses (GenBank Accession Nos. DQ000175–DQ000195 and DQ002477–DQ002479).

Since the pericentric inversion breakpoints of the chimpanzee and bonobo chromosomes homologous to *HSA* 16 (Fig. 4E) appear to be identical, the FISH pattern of the breakpoint-spanning BACs RP43-007E19 and RP43-001I03 may be explicable in terms of a duplication that occurred exclusively in the bonobo lineage (Fig. 3). Thus, in addition to the pericentric inversion of the bonobo chromosome homologous to *HSA* 16, a bonobo-specific duplication appears to have occurred close to the breakpoint sites.

Discussion

Among the extant hominoids (great apes), the orangutan arose in East Asia 11–16 Mya and diverged under allopatric

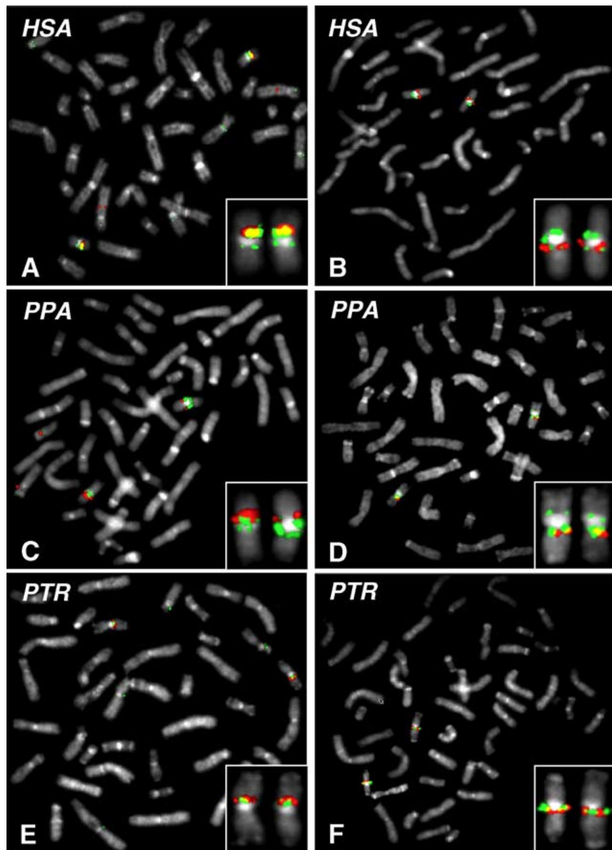

Fig. 2. *HSA, PPA*, and *PTR* metaphases after hybridization with BACs from the chromosome homologous to *HSA* 16. (A, C, E) Cohybridization of *PTR* p-arm breakpoint-spanning BAC RP43-007E19 (green) and *HSA* p-arm indicating BAC RP11-347N4 (red) to (A) *HSA*, (C) *PPA*, and (E) *PTR* chromosomes. (B, D, F) Cohybridization of *PTR* q-arm breakpoint-spanning BAC RP43-00I03 (green) and *HSA* q-arm indicating BAC RP11-46D6 (red) to (B) *HSA*, (D) *PPA*, and (F) *PTR* chromosomes. The split signals of *PTR* BACs RP43-007E19 and RP43-00I03 on *PPA* chromosomes homologous to *HSA* 16 are indicative of a duplication that occurred after the separation of the *PTR* and *PPA* lineages.

conditions with respect to the other hominoid species. By contrast, the gorillas, chimpanzees, and humans arose parapatrically in East Africa and became separated from one another within the past 8 million years [1,23].

Comparative chromosome analysis of the hominoids using Old World monkeys as an outgroup has indicated that orangutans and humans have retained a karyotype that is much closer to the ancestral hominoid condition than those of the chimpanzee or gorilla [6,24,25]. The karyotypes of the species, which diverged under parapatric conditions (i.e., gorilla and chimpanzee), show more derived changes than do those species that diverged under allopatric conditions. This is reminiscent of chromosomal speciation in *Drosophila*. Those *Drosophila* species that are sympatric with their sister species differ from them by inversions, in contrast to the closely related species that do not co-occur geographically [26].

Among the nine pericentric inversions that distinguish the karyotypes of humans and chimpanzees, seven became fixed in the chimpanzee lineage. They affect the homologs of *HSA* 4, 5, 9, 12, 15, 16, and 17. We have demonstrated in this study that in six of these inversions, the breakpoints occur at the same nucleotide positions in the two extant species of chimpanzee: the common chimpanzee and the bonobo (Fig. 4). In the case of the seventh inversion, which affects the chimpanzee homologue of *HSA* 15, the pericentric inversion breakpoint was mapped both in *PTR* and in *PPA* to the region consisting of segmental duplications [22]. The occurrence of identical inversions in both chimpanzee species has not previously been demonstrated and requires sequence comparisons of the breakpoint regions. There are many instances of chromosomal rearrangements occurring recurrently at approximately the same position during chromosome evolution. As an example, the pericentric inversions of chimpanzee and gorilla chromosomes homologous to human chromosome 16 have breakpoints in the same regions as determined by the same breakpoint-spanning BAC; however, at the DNA sequence level the breakpoints turned out not to have occurred at identical sites

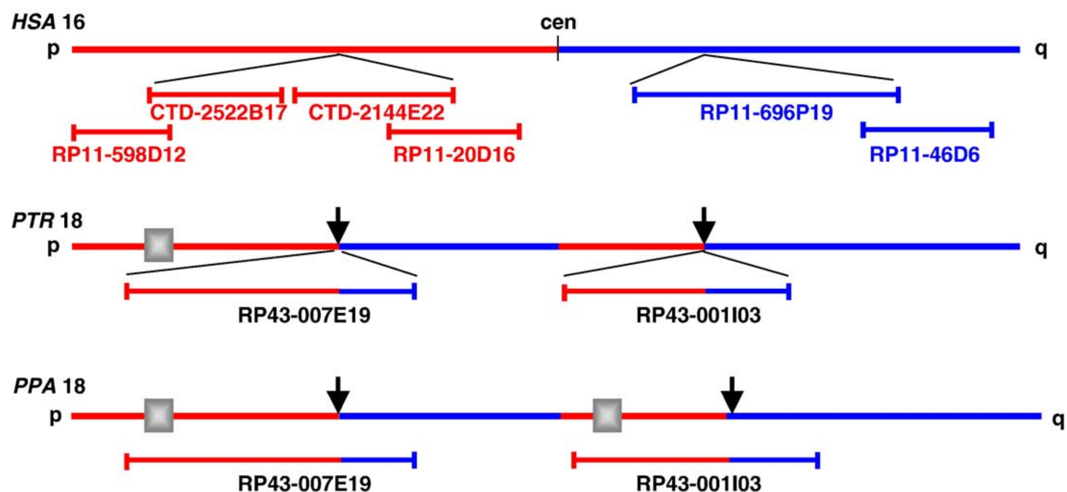

Fig. 3. Illustration of the pericentric inversion distinguishing *HSA* 16 and the homologous *PTR* and *PPA* chromosomes. The breakpoint-spanning and neighboring BACs are indicated by horizontal bars. Duplicated signals of *PTR* BACs RP43-007E19 and RP43-001I03 on bonobo chromosome 18 (*PPA* 18) are due to the duplication of a segment (gray rectangle) that occurred exclusively in the bonobo lineage.

Table 3
Results of the junction-PCR analyses of the breakpoint-spanning fragments

Chromosome	Primer sequences (5′ → 3′)	Primers designed from the sequence of	Primer positions on the breakpoint-spanning BAC	Primer positions on the *PTR* sequence scaffold[a]	Size of PCR product (bp)	Amplification in		
						HSA	*PTR*	*PPA*
PTR 3p	TCTTTTGTCTTTGTCTGTCTGGA	RP43-59P20	3F: 2108	Chromosome_3 3F: 47357089	1393[b]	–	+	+
(*HSA* 4)	CCAGAAATAGGGACAGGTGAA	(AY335550)	3R: 3501	Chromosome_3 3R: 47358578				
PTR 4p	AAACAAATATCCACCCACAACC	Scaffold *PTR*	—	Chromosome_4 4F: 19219378	993	–	+	+
(*HSA* 5)	CATTCCCACTCACAATGCTC	Chromosome 4	—	Chromosome_4 4R: 19220371				
PTR 11p	CAAATACATTTTTGTCACTATTTGTCA	RP43-163C1	11F: 145549	11_random 11F: 20572533	1921	–	+	+
(*HSA* 9)	TGCTCCAAATGTCTCCCAGT	(AY569337)	11R: 147468	11_random 11R: 20574454				
PTR 10q	CCTTGACTGGCTCTTCCACT	RP43-135M19	10F: 147897	Un_random 10F: 47210210	226	–	+	+
(*HSA* 12)	GGACACTGGATATCTCACATGG	(AC007214)	10R: 148123	Un_random 10R: 47210436				
PTR 18q	TGGGTGAGAGTTTTCCAAGC	Scaffold *PTR*	—	Un_random 18F: 8823489	197	+[c]	+	+
(*HSA* 16)	CTACTGGCACTGGACTTCAGC	Un_random	—	Un_random 18R: 8823686				
PTR 19q	ACCCTTTAGATCAGAAGAATCCTG	RP43-134L13	19F: 7895	19_random 19F: 30581404	340	–	+	+
(*HSA* 17)	AAAAACCTGATCCCGTAGGC	(AY117035)	19R: 8235	19_random 19R 30581744				

[a] According to the draft sequence of the chimpanzee genome (Ensembl, release 27.1a.1).
[b] The size of the PCR product corresponds to the difference between primer positions on the breakpoint-spanning BAC RP43-59P20 but not to the difference between the primer positions on the *PTR* draft sequence scaffold, owing to the unfinished status of this *PTR* sequence.
[c] The PCR product amplified from *HSA* genomic DNA was false positive and exhibited high similarity to sequences on *HSA* 2, 14, and 22.

[20]. Furthermore, breakpoint heterogeneity has also been observed in de novo pericentric inversions in humans, which are cytogenetically very similar [27,28].

The presence of the same seven inversions in the common chimpanzee and the bonobo renders it unlikely that they represent presence/absence variation among the subspecies of the common chimpanzee. Population studies of the common chimpanzee have revealed the existence of several distinct subspecies: *P. troglodytes troglodytes, P. troglodytes verus*, and *P. troglodytes schweinfurthii* [29]. The nucleotide diversity between these subspecies is 0.132%, which is little more than a third of the divergence between the common chimpanzee and the bonobo, which amounts to 0.373% [30]. Most likely, the longer separation time between the bonobo and the common

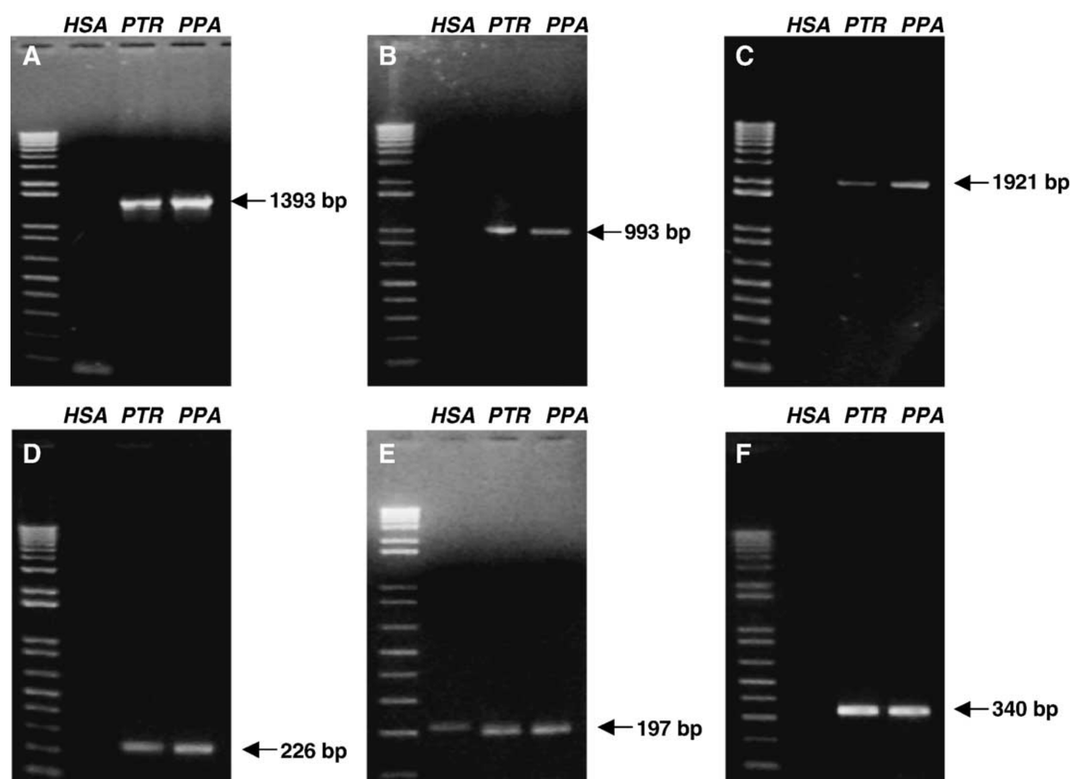

Fig. 4. Junction-PCR analysis of the breakpoints. Breakpoint-spanning fragments of regions homologous to (A) *HSA* 4, (B) *HSA* 5, (C) *HSA* 9, (D) *HSA* 12, (E) *HSA* 16, and (F) *HSA* 17. Positive junction-PCR products were obtained in *PTR* and *PPA* but not in *HSA*. The only exception was the product amplified from the inversion junction of *PTR* 18, homologous to *HSA* 16, from which a PCR product of atypical length was obtained using human genomic DNA as a template (E) but was subsequently found by sequence analysis to be a false positive. The sizes of the respective PCR products are indicated on the right.

chimpanzee than between the common chimpanzee subspecies is responsible for these differences.

Our findings indicate that the chimpanzee inversions are identical by descent rather than recurrent and that they predate the separation of the two chimpanzee species [3,4]. It would thus appear that at least six of the nine inversions were early acquisitions during chimpanzee evolution, such that some or all might have been present at the time of speciation, a process that permanently separated the chimpanzee and human lineages. This is important since the inversions could themselves have promoted speciation.

The classical model of chromosomal speciation predicts reproductive isolation caused by reduced fertility of hybrids between chromosomally different subpopulations. The reduced fitness of hybrid progeny (owing to heterozygosity in chromosomal rearrangements causing meiotic disturbances) can thus lead to speciation either as a result of reduced gene flow between the populations or by the promotion of assortative mating [31,32]. One problem for this classical model is, however, that if the fitness costs of the rearrangements are assumed to be sufficiently high to promote speciation, it may be difficult for these rearrangements to become fixed in the respective populations. Recently, a new model of chromosomal speciation has been proposed that contrasts with previous ideas regarding the role of chromosomal rearrangements in speciation, since it assumes that the inversions exert only a minimal influence on fitness and hence have a high likelihood of becoming fixed in the population. This new model of chromosomal speciation relies upon recombination suppression, an idea already put forth in various forms [26,32–34]. According to this chromosomal speciation model, the inversions may have acted as potential drivers of the speciation process that led to the permanent separation of the human and chimpanzee lineages. It predicts that in heterozygotes, the chromosomal rearrangements act as barriers to gene flow by virtue of their ability to suppress recombination. This is then postulated to lead to an accumulation of genetic incompatibilities, reproductive isolation, and finally speciation.

The relevance of the pericentric inversions to the process of speciation between the ancestors of extant humans and chimpanzees has been tested by comparing the DNA divergence rates between rearranged and collinear chromosomes. Those chromosomes that carry rearrangements between humans and chimpanzees exhibited a higher level of divergence in coding regions than did their counterparts in nonrearranged chromosomes [8]. However, these observations have not been reproduced in subsequent studies [13,14].

In any attempt to identify differences in DNA divergence rates, it is vital to be able to demarcate the boundaries of the inversions accurately at the DNA sequence level. Since the new model of chromosomal speciation requires that recombination is suppressed in inverted regions yet retained in noninverted regions of the same chromosome, it is important to delimit precisely the inversions from the noninverted portion of the chromosome. This has not invariably been taken into account in the aforementioned studies [8,13,14]. The analyses performed here have served to demarcate these inversions very precisely. Interestingly a trend toward increased gene expression divergence in rearranged chromosomes has been observed and this trend seems not be caused by local effects of close chromosomal breakpoint [15,35].

Whether or not there is a relationship between the inversions and speciation, it is clear that a large number of chromosomal rearrangements of the same general type (pericentric inversions) have become fixed in the ancestors of the bonobo and common chimpanzee within a relatively short period of time (3 million years). Further, different inversions (and only one translocation) have been fixed in the gorilla lineage in approximately the same time period. This phenomenon of repeated and rapid occurrence of the same type of chromosomal rearrangement in a given lineage has been commonly reported in studies of chromosomal evolution and has been termed “karyotypic orthoselection” [36]. The basis of karyotypic orthoselection is, however, not well understood and further work is needed to elucidate the biological role of the chromosomal inversions during human/chimpanzee speciation.

Material and methods

Cell lines and FISH

The two bonobo lymphoblastoid cell lines used in these studies were established from peripheral blood samples of two females from the Frankfurt Zoo and were a generous gift from Dr. Ulrich Zechner (Mainz, Germany). The lymphoblastoid cell line *PTR*-EB176 (ECACC No. 89072704), derived from common chimpanzee, was purchased from the European Collection of Cell Cultures (www.ecacc.org.uk). Two lymphoblastoid cell lines from human donors were investigated. Metaphase spreads were prepared from these samples according to standard procedures and DNA was extracted using the DNeasy isolation kit (Qiagen).

BAC and PAC clones listed in Tables 1 and 2 were purchased from the BACPAC Resource Center (www.chori.org/bacpac). BAC/PAC DNA was isolated using the Qiagen Midi-Kit and used as FISH probes. At least 1 μg BAC DNA was labeled either with biotin-16–dUTP (Roche Diagnostics) and detected with FITC–avidin and biotinylated anti-avidin (Vector) or labeled with digoxigenin-11–dUTP (Roche Diagnostics) and detected by mouse anti-digoxigenin. In a second step, anti-mouse antibodies coupled with Texas red and produced in rabbit were used, followed by anti-rabbit antibodies conjugated with Texas red (Dianova). Slides were counterstained with diamidinophenylindole and mounted with Vectashield antifade solution (Vector).

Junction-PCR analyses of the breakpoint-spanning fragments

Breakpoint regions were analyzed by PCR using primers that were designed to amplify fragments across the inversion breakpoint junctions. The primers were designed by reference to the chimpanzee sequence represented either by the draft sequence of the chimpanzee genome (Ensembl release 27.1a.1) or by BAC clones that were isolated and sequenced during the previous characterization of the inversion breakpoints [7,17,19]. The primers used for these analyses are listed in Table 3.

End-sequencing of breakpoint-spanning fragment

The sequences of the breakpoint-spanning fragments were determined from both ends using the BigDye Terminator v.3.1 Cycle Sequencing Kit. The analyses were performed using an ABI Prism 3100 sequencer (Applied Biosystems). Alignments and homology searches were performed with the BLAST program at the NCBI server (www.ncbi.nlm.nih.gov) and FASTA analyses using the Wisconsin Package version 10.2, Genetics Computer Group.

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