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Refinement of macaque synteny arrangement with respect to the official rheMac2 macaque sequence assembly

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Abstract

We have compared the synteny block organization of the official macaque genome sequence assembly (Jan. 2006; rheMac2) with an independent assembly that used a molecular cytogenetic approach. The mapping of four synteny segments, ranging in size from 4 Mb to 24 Mb, was found to be inconsistent between the two datasets. We specifically investigated these discrepancies by appropriate co-hybridization FISH experiments with validated reference probes located outside the area under study. We found that in the macaque rheMac2 release three synteny segments were wrongly mapped and one segment was incorrectly oriented.

Abbreviations

BAC	bacterial artificial chromosome
BTA	*Bos taurus*
CAE	*Cercopithecus aethiops*
ChIP	chromatin immunoprecipitation
CJA	*Callithrix jacchus*
CMO	*Callicebus moloch*
Cy	cyanine
DAPI	4′,6-diamidino-2-phenylindole
ENC	evolutionarily new centromeres
FISH	fluorescence *in-situ* hybridization
GA	great apes ancestor
GGO	*Gorilla gorilla*
HSA	*Homo sapiens*
LCA	*Lemur catta*
MMU	*Macaca mulatta*
NCBI	National Center for Biotechnology Information
NWM	New World monkey
OWM	Old World monkey
PA	primate ancestor
PCR	polymerase chain reaction
PPY	*Pongo pygmaeus*
PTR	*Pan troglodytes*
RP	Roswell Park
SBO	*Saimiri boliviensis*
TCR	*Trachypithecus cristatus*
UCSC	University California Santa Cruz

Introduction

The human genome is the only vertebrate genome whose sequencing was achieved using a solely 'hierarchical' shotgun approach, consisting of the selective sequencing of previously ordered genomic clones (Lander *et al*. 2001, McPherson *et al*. 2001, Dobigny *et al*. 2005). The pure shotgun method is less time- and money-consuming, but the risk of misassembly is usually considered higher than with the hierarchical approach (Green 1997).

The macaque genome (*Macaca mulatta*, MMU) was sequenced and assembled using, essentially, the shotgun method (Gibbs *et al*. 2007). An independent synteny block arrangement in macaque has been established using a molecular cytogenetic approach (FISH synteny assembly, FISH-SA) (Ventura *et al*. 2007). The results of this study, which used a panel of about 500 human BACs, are graphically displayed at the Web site www.biologia.uniba.it/macaque/, as supplementary material to the publication. A detailed comparison of the FISH-SA with the official macaca sequence assembly (rheMac2; Jan. 2006) as reported

by UCSC browser, disclosed some inconsistencies. We investigated these discrepancies by specific FISH experiments using appropriate human BAC clones. We also considered the large amounts of data on macaque genome organization already published and obtained using different approaches: cytogenetics using chromosome painting (Wienberg *et al*. 1992, Moore *et al*. 1999), radiation hybrids (Murphy *et al*. 2005), and linkage (Rogers *et al*. 2006).

The results, first, identified some inappropriately-localized centromeres and unconventionally oriented chromosomes within rheMac2. More significantly, comparison of the maps revealed four DNA segments, ranging in size from 4 Mb to 24 Mb, wrongly mapped or wrongly oriented in the rheMac2 assembly.

Materials and methods

Metaphase preparations were obtained from lymphoblastoid or fibroblast cell lines of the following species: common chimpanzee (*Pan troglodytes*, PTR); gorilla (*Gorilla gorilla*, GGO); Borneo orangutan (*Pongo pygmaeus pygmaeus*, PPY); OWM— rhesus monkey (*Macaca mulatta*, MMU, Cercopithecinae); Barbary macaque (*Macaca sylvanus,* MSY, Cercopithecinae); African green monkey (*Cercopithecus aethiops*, CAE, Cercopithecinae); silvered leaf monkey (*Trachypithecus cristatus*, TCR, Colobinae); NWM—woolly monkey (*Lagothrix lagothricha*, LLA, Atelinae); common marmoset (*Callithrix jacchus*, CJA, Callitricinae); dusky titi (*Callicebus moloch*, CMO, Callicebinae); squirrel monkey (*Saimiri boliviensis boliviensis*, SBO, Callicebinae); Strepsirhini—ring-tailed lemur (*Lemur catta*, LCA, Lemurinae).

All these cell lines, with the exception of lemur, are lymphoblastoid cell lines obtained by Epstein–Barr virus transformation. Alteration of the karyotype is occasionally observed in transformed cell lines. We used these cell lines extensively and rearrangements were never observed. Rearrangements are easily noted because they are always at least initially present in a heterozygous state. The karyotype of the macaque cell line used here is shown at our Web page http://www.biologia.uniba.it/macaque/MMU/MMU_kar.html.

DNA extraction from BACs was reported previously (Ventura *et al*. 2001). Co-hybridization FISH experiments were performed essentially as described by Lichter *et al*. (1990). Digital images were obtained using a Leica DMRXA2 epifluorescence microscope equipped with a cooled CCD camera (Princeton Instruments, Princeton, NJ, USA). Cy3-dCTP, fluorescein-dCTP, Cy5-dCTP and DAPI fluorescence signals, detected with specific filters, were recorded separately as greyscale images. Pseudocolouring and merging of images were performed using Adobe Photoshop software.

Results

An accurate comparison, by visual inspection, of the FISH-SA data (www.biologia.uniba.it/macaque) with the macaque rheMac2 sequence release (Jan. 2006; http://genome.ucsc.edu) revealed four inconsistencies affecting regions larger than 3 Mb. These are listed in Table 1. Three consisted in the wrong mapping of synteny blocks (two on chromosome 1 and one on chromosome 13). The fourth discrepancy involved a segment of 20 Mb incorrectly oriented.

In evaluating the orientation of synteny segments in macaque with respect to humans, it is important to note the following. Chromosomes are usually represented with the short arm on ‘top’ and, for each human chromosome, the base-pair count conventionally starts from the tip of the short arm. In several

Table 1. Inconsistencies between rheMac2 and FISH-SA assemblies

Human (hg18) synteny segment	Size in HSA (kb)	Markers	Macaque	
			rheMac2	FISH-SA
chr1:161,033,866–177,521,905	16 448	N-Q	Different position	
chr4:36,549–3,852,865	3 818		MMU1	MMU5
chr2:114,076,736–138,830,121	24 753		MMU13	MMU12
chr1:226,810,735–246,932,000	20 121	(V)W-Z	Reverse	Forward

macaque chromosomes the centromere index presented in the macaque release was incorrect because of rearrangements or simply because of centromere repositioning events. The latter phenomenon affected nine macaque chromosomes (Ventura *et al*. 2007). The sequence orientation adopted in the rheMac2 release, however, did not take into account these changes, and used the human sequence as a reference,

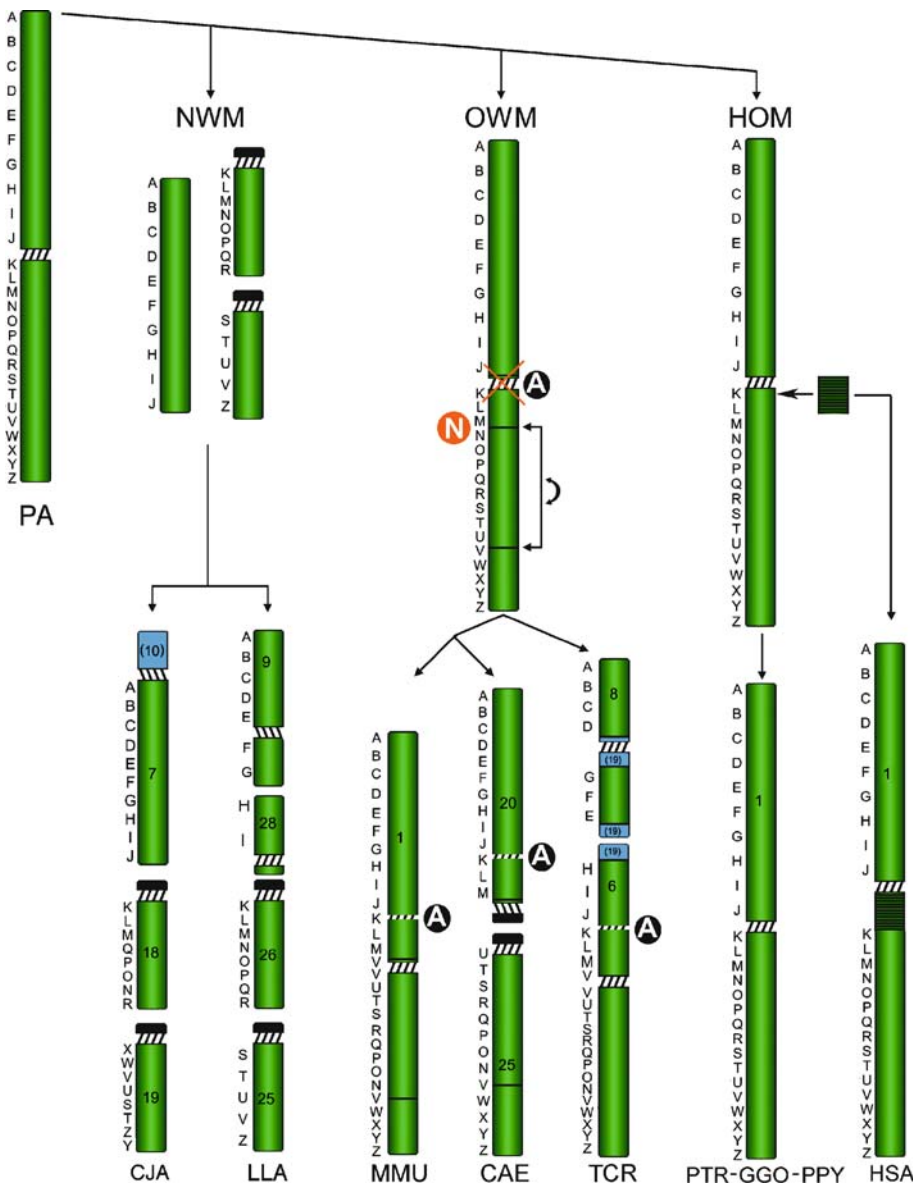

Figure 1. Diagrammatic representation of the evolutionary history of chromosome 1 in primates, reconstructed taking into account also the evolutionary history of chromosome 1 in non-primate mammals (Murphy *et al*. 2003). N in a red circle stands for new centromere. A in a black circle stands for the ancestral centromere. Letters on the left refer to the markers reported in Table 2. NWM stands for New World monkeys; OWM for Old World monkeys; HOM for Hominoidea; PA for primate ancestor. The studied species (3-letter abbreviations) are reported in Materials and methods. The centromere on the rheMac2 assembly cannot be derived from the sequence, but for clarity was annotated along with the position of the ancestral centromere. The arrows delimit the large inversion region detected by the FISH-SA. Macaque chromosome 1 is upside-down to facilitate comparison. Regions in blue are contributed by a different human chromosome, reported in parentheses. Novel centromeres that emerged in New World monkeys are not annotated.

Table 2. Human BAC clones used to track HSA1 evolution

Code	Probe	Acc. no.	hg18 (UCSC March 2006)	Map
A	RP11-421C4	AC026283	chr1_random:195,187–392,934	1p36.33
B	RP11-265F14	AL512883	chr1:15,630,693–15,735,006	1p36.21
C	RP11-266K22	AL451070	chr1:31,512,242–31,645,345	1p35.2
D	RP11-492I2	AL583843	chr1:50,340,248–50,402,510	1p33
E	RP11-55M23	ends	chr1:55,214,018–55,384,910	1p32.3
F	RP11-316C12	AL627317	chr1:71,621,580–71,715,216	1p31.1
G	RP11-254E16	BES	chr1:84,539,796–84,689,450	1p31.1
H	RP11-138K16	AC093559	chr1:99,728,340–99,904,318	1p21.2
I	RP11-284N8	AL365361	chr1:110,897,276–111,090,458	1p13.3
J	RP11-192J8	BES	chr1:118,169,383–118,169,829	1p12
K	RP11-35B4	AL359093	chr1:120,333,471–120,334,001	1p12
HSA centromere				
K	RP11-35B4	AL359093	chr1:144,000,592–144,166,607	1q21.1
L	RP11-98F1	AL353760	chr1:153,539,660–153,543,654	1q22
M	RP11-572K18	AL445197	chr1:160,918,751–161,035,790	1q23.3
N	RP11-165D2	BES	chr1:161,820,136–161,965,025	1q23.3
O	RP11-;26I16	BES	chr1:162,132,728–162,280,652	1q23.3
P	RP11-63N10	BES	chr1:172,808,817–172,961,245	1q25.1
Q	RP11-101H2	BES	chr1:174,809,204–174,971,554	1q25.2
R	RP11-134C1	BES	chr1:186,252,053–186,417,889	1q31.1
S	RP11-553K8	AL157402	chr1:196,750,597–196,960,927	1q31.3–1q32.2
T	RP11-57I17	AL137789	chr1:205,811,787–205,957,118	1q32.2
U	RP5-915N17	AL139288	chr1:226,661,126–226,848,488	1q42.13
V	RP4-621O15	AL713899	chr1:226,810,735–226,866,653	1q42.13
W	RP4-646B12	AL096776	chr1:226,864,654–226,955,397	1q42.13
X	RP11-3K22	AL359874	chr1:227,082,837–227,162,782	1q42.13
Y	RP11-385F5	AL359921	chr1:234,752,824–234,966,581	1q43
Z	RP11-438F14	AC098483	chr1:246,754,133–246,932,000	1q44

probably to facilitate comparison. In the FISH-SA, in contrast, the macaque chromosomes are oriented in the classical cytogenetic way (www.biologia.uniba.it/macaque/).

Comparison of marker order arrangement in different primate species allows one to track the flow of changes that a chromosome has undergone during evolution. The complex synteny patchwork detected on macaque chromosome 2 (human 3; www.biologia.uniba.it/macaque/MMU/MMU_02.html), for instance, can be fully understood only in the light of the evolutionary history of this chromosome; three independent inversions occurred from the last Hominoidea–Old World monkeys (OWM) common ancestor, one in the lineage leading to macaque, and two in the lineage leading to humans (Ventura *et al*. 2004). To better interpret the synteny discrepancies we have detected on macaque chromosome 1 (HSA1) and 12 (HSA2q), we refined, with respect to the work already done by Stanyon *et al*. (2008), the evolutionary history of these two chromosomes in primates, using new appropriate BAC clones. The results are graphically summarized in Figure 1. BAC clones used as markers are listed in Table 2. The global alignment of human and macaque chromosome 1 masked sequences was performed using the GenAlyzer program (an improved version of Reputer software) according to the authors' instructions (Kurtz *et al*. 2001) (http://www.genomes.de/). The graphical output is reported in Figure 2. The evolution of human chromosome 2q is illustrated in Figure 3; probes are listed in Table 3.

In order to solve the discrepancies reported above, appropriate human BAC clones were chosen on the UCSC browser (hg18 assembly, UCSC March 2006 release) and first used in FISH experiments on human metaphases to validate their mapping. The validated BACs (Table 4) were then used in FISH experiments in macaque. In each experiment, the test probes were co-hybridized with probes mapping outside the controversial area, used as a reference for unambiguous chromosome identification and/or segment

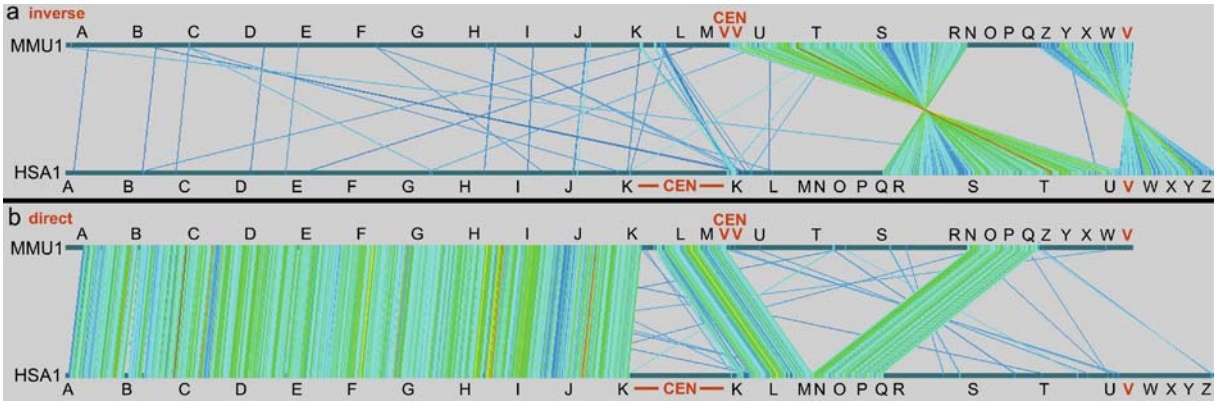

Figure 2. Wide-view graphic results of MMU (upper line) and human (lower line) chromosome 1 using the GenAlyzer program (see text). Inverse (**a**) and direct (**b**) orientations are displayed separately. The letters, defining the synteny blocks, are reported in Table 2. To reduce background, only stretches longer than 200 bp are shown. Colours refers to the length of paired segments (red = maximum). The HSA1 line appears significantly longer because the large gap corresponding to the centromere (flanked by K marker in the figure) is absent in macaque. The tip of the macaque chromosomes is supposed, in rheMac2, to be a portion of the tip of the human chromosome 4 (see text and Figure 4d).

orientation. Relevant results are summarized in Figure 4, which also reports ideograms of chromosome 1 according to the FISH-SA and rheMac2 assemblies (Figure 4a). Figure 4f shows a FISH experiment performed using the BAC clone RP4–621O15 (marker V) which yielded signals, on MMU1, at both breakpoints of the large inversion identified by the FISH-SA, and a third FISH signal at the opposite side of the MMU1 centromeric region. In all cases the results were unequivocal and supported the conclusion that the four segments reported in Table 1 were wrongly mapped or oriented in the rheMac2 assembly (see legend of Figure 4 for details). To further confirm our conclusions, we performed the same FISH experiments in two additional unrelated macaque individuals and on an additional macaque species, the tailless macaque (*Macaca sylvana*). All the results perfectly matched those obtained on the first macaque individual (data not shown).

Szamalek *et al*. (2006) reported a human-specific pericentric inversion involving the heterochromatic block of chromosome 1. We did not investigate this inversion because it essentially involved only the centromeric satellite DNA block.

Discussion

As is widely known, the pure shotgun approach can result in misassembly of difficult regions of a genome. Usually these misassemblies are the consequence of problems in joining sequence contigs (scaffolds) when their borders fall within regions rich in segmental duplications. The shortcomings of the shotgun assembly process can often be resolved by comparing the sequence assembly with data obtained using a cytogenetic approach to quickly and easily test alternative hypotheses. The cytogenetic approach is independent of the sequence-based assembly and, at least in relatively close species like macaque and humans, is facilitated by the fact that human clones hybridize relatively well in macaque and vice versa. Molecular cytogenetics can therefore be advantageously exploited in resolving assembly uncertainties, as already illustrated by Rocchi *et al*. (2006).

The present FISH-based study on the evolutionary history of chromosome 1 (see Figure 1), coupled with the available data on the evolution of this chromosome in non-primate mammals (Murphy *et al*. 2003), indicated that the human form very likely represents the ancestral primate form of this chromosome for both marker order and centromere position. An inversion of a segment, corresponding in humans to chr1:161.0 Mb–226.9 Mb, is shared by *Macaca mulatta* (Cercopithecinae) and silvered leaf-monkey (*Trachypithecus cristatus*, Colobinae). The position of the centromere, also shared by Cercopithecinae and Colobinae (see Figure 1), is at variance with respect to humans because of a centromere repositioning event (Ventura *et al*. 2007). Very likely, therefore, both the centromere move and the

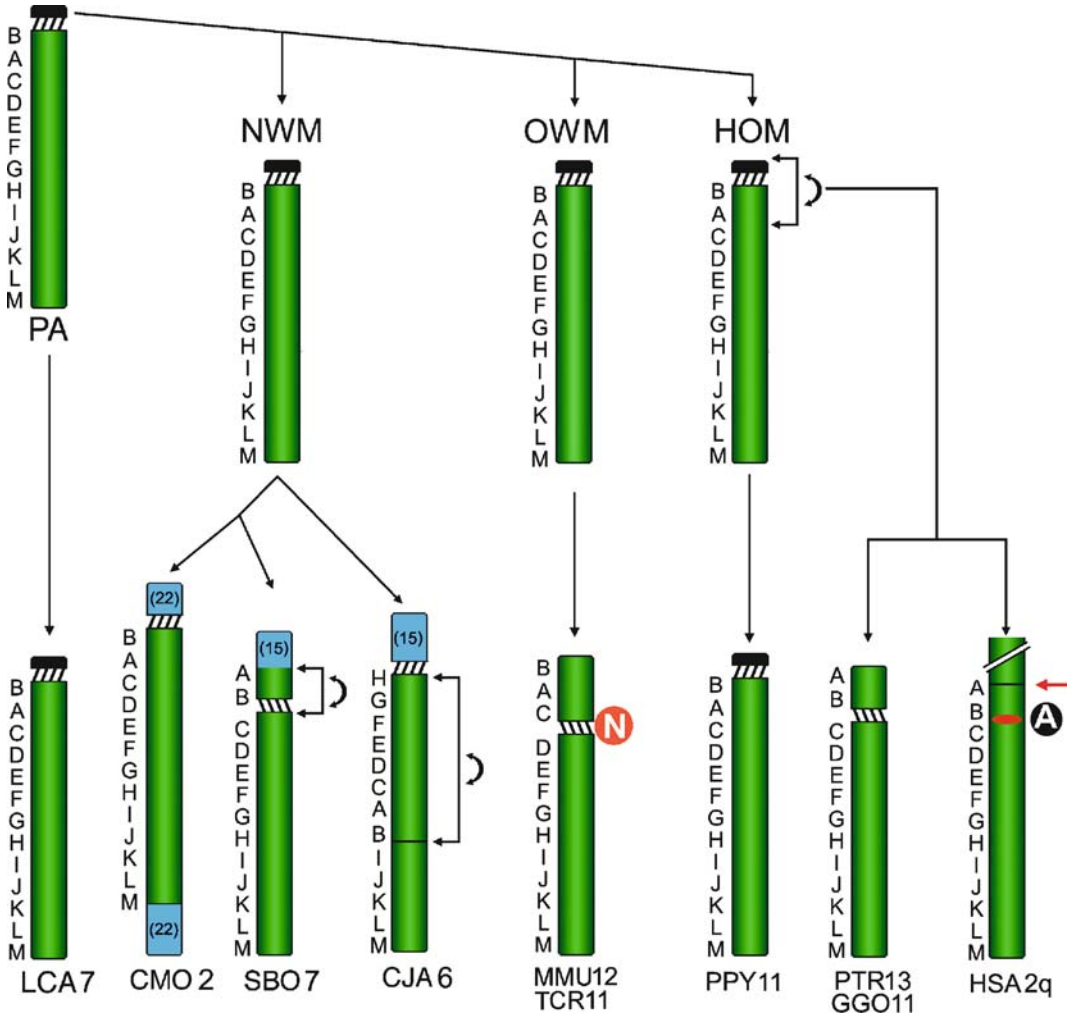

Figure 3. Diagrammatic representation of the evolutionary history of chromosome 2q in primates. N in a red circle stands for new centromere. A in a black circle stands for the ancestral centromere that inactivated following the well-known human-specific IIq/IIp fusion. The red arrow indicates the fusion point. Letters on the left refer to the markers reported in Table 3. NWM stands for New World monkeys; OWM for Old World monkeys; HOM for Hominoidea. The studied species (3-letter abbreviations) are reported in Materials and methods. Regions in blue are contributed by a different human chromosome, reported in parentheses. Novel centromeres that emerged in New World monkeys are not annotated.

chromosomal inversion occurred in Old World monkeys' ancestor. The domain where the centromere repositioned corresponds to the proximal breakpoint of the inversion. The seeding of evolutionarily novel centromeres or human clinical neocentromeres at breakpoint regions has already been noted (Ventura *et al*. 2003, 2004, Marshall *et al*. 2008). It is worth noting, in this respect, that the BAC clone RP4-621O15 (marker V), located, in humans, at the distal breakpoint of the inversion, was duplicated at the proximal break of the inversion (that is at the side of the centromere facing the inversion) (Figure 4f). Then, very likely, it was further duplicated on the other side of the centromere (Figure 4f), as a consequence of the pericentromeric duplication activity typical of these regions (Ventura *et al*. 2007). This triplication probably contributed to the misassembly of the segments defined by markers N→Q and (V)W→Z (Figures 2 and 4).

The translocation involving chromosomes 1 and 5 (human 4) did not match our reconstruction of the evolutionary history of chromosome 1, and was not

Table 3. Human BAC clones used to track HSA2q evolution

Code	Probe	Acc. no.	hg18 (UCSC March 2006)	Map
A	RP11-321K13	AC012449	chr2:117,348,561–117,457,895	2q14.1
B	RP11-521O16	AC012508	chr2:127,449,511–127,635,477	2q14.3
C	RP11-34L23	AC011893	chr2:136,170,022–136,347,922	2q21.3
D	RP11-107E 5	AC009951	chr2:144,881,008–145,040,798	2q22.3
E	RP11-357O18	AC010730	chr2:155,061,766–155,227,716	2q24.1
F	RP11-361O8	AC019197	chr2:165,429,457–165,607,711	2q24.3
G	RP11-504O20	AC016751	chr2:176,383,226–176,559,604	2q31.1
H	RP11-335G13	AC097500	chr2:186,490,013–186,670,227	2q32.1
I	RP11-449J2	AC013274	chr2:196,210,882–196,336,014	2q32.3
J	RP11-325M10	AC007385	chr2:206,054,358–206,242,419	2q33.3
K	RP11-804M4	AC073284	chr2:215,843,859–215,940,486	2q35
L	RP11-348M14	BES	chr2:226,668,369–226,840,985	2q36.3
M	RP11-463B12	AC013469	chr2:240,559,949–240,731,368	2q37.3

supported by the evolutionary history of chromosome 4 reported by Marzella *et al*. (2000). The specific experiments reported in Figure 4d definitively excluded the translocation.

It is well known that human chromosome 2 is the result of a human-specific telomere–telomere fusion of two ancestral acrocentric chromosomes (Yunis & Prakash 1982). The two stretches of degenerated telomeric sequences located at chr2:114,076,792–14,077,524 (2q14.1) and arranged head-to-head, clearly document this fusion (Ijdo *et al*. 1991). In the absence of subsequent inversions encompassing this fusion point (Stanyon *et al*. 2008), all segments centromeric to this boundary are expected to map on the evolutionary IIp, while sequences telomeric to it are expected on IIq. In macaque, the two IIp and IIq chromosomes correspond to MMU13 and MMU12, respectively. The rheMac2 mapped

Table 4. Human BACs used to discriminate the inconsistencies

Probe	Acc. no.	hg18 (UCSC March 2006)	Map
HSA1			
RP11-344E20	BES	chr1:22,459,769–22,647,562	1p36.12
RP11-165D2	BES	chr1:161,820,136–161,965,025	1q23.3
RP11-101H2	BES	chr1:174,809,204–174,971,554	1q25.2
RP11-3K22	AL359874	chr1:227,082,837–227,162,782	1q42.13
RP11-385F5	AL359921	chr1:234,752,824–234,966,581	1q43
RP4-621O15	AL713899	chr1:226,810,735–226,866,653	1q42.13
HSA2			
IIp (MMU13)			
RP11-3A17	BES	chr2:54,836,724–54,997,743	2p16.1
RP11-419E14	BES	chr2:78,739,741–78,929,009	2p12
RP11-519H15	AC005040	chr2:106,847,336–107,037,284	2q12.3
IIp/IIq fusion point[a]		chr2:114,076,792–14,077,524	2q14.1
IIq (MMU12)			
RP11-432G15	BES	chr2:114,091,187–114,285,568	2q14.1
RP11-348M14	BES	chr2:226,668,369–226,840,985	2q36.3
HSA4			
RP11-42F9	BES	chr4:1,622,433–1,783,949	4p16.3
RP11-529E10	AL590235	chr4:3,384,158–3,543,543	4p16.2
RP11-1142B14	BES	chr4:4,265,167–4,423,249	4p16.3
RP11-264P23	BES	chr4:120,689,651–120,841,110	4q27

[a]The fusion point is defined by two stretches of telomeric sequences, arranged head-to-head, as reported by Ijdo *et al*. (1991).

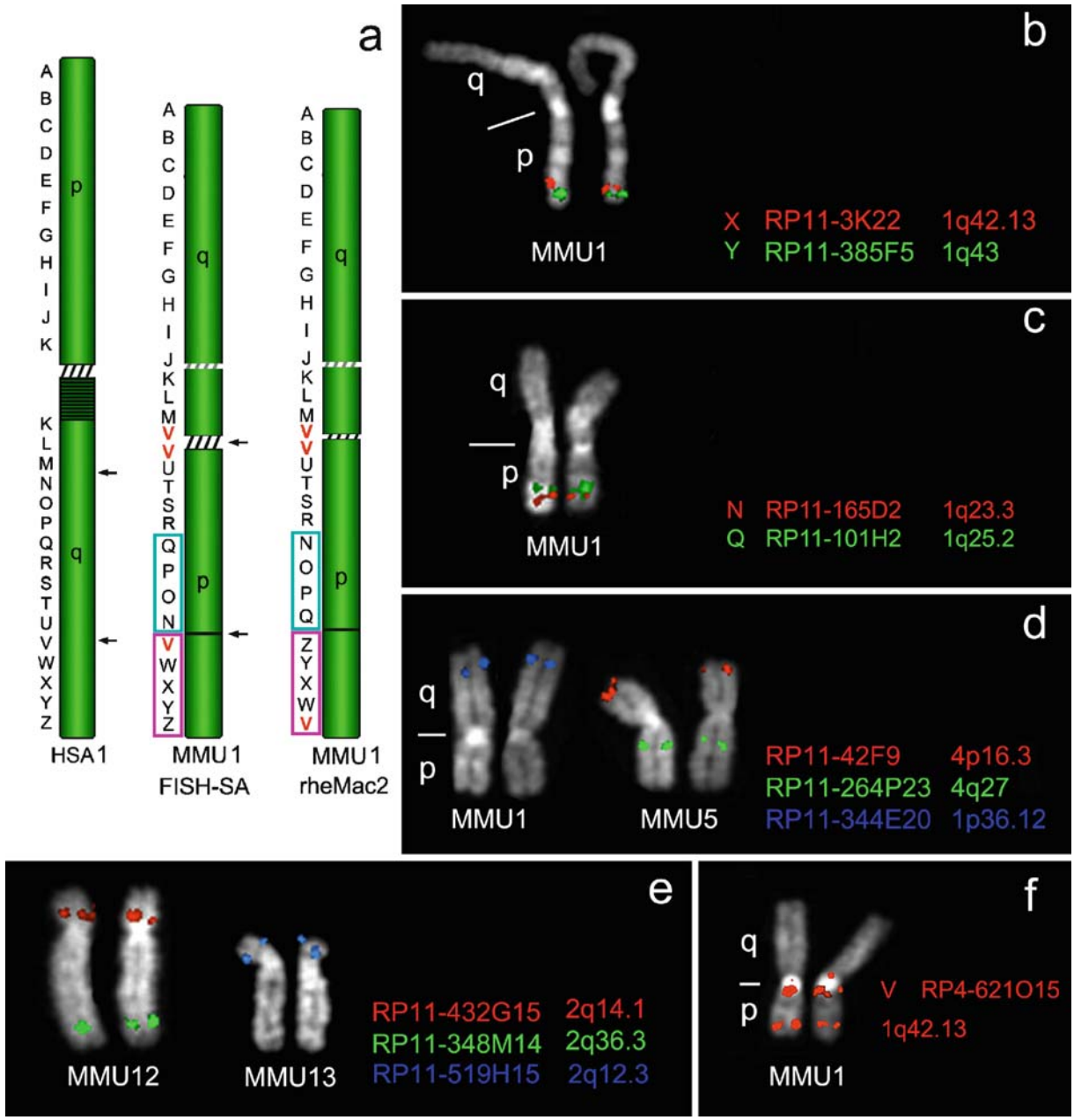

Figure 4. Results of relevant FISH experiments aimed at discriminating the inconsistencies between the rheMac2 and FISH-SA assemblies. Note that the macaque chromosome 1 (MMU1) is upside-down with respect to the relative p and q arm ratio to facilitate comparison (the MMU1 arm ratio was altered because of the absence of the large, human-specific, 1q12 heterochromatic block and of the OWM-specific centromere repositioning event; see text). (**a**) Differences in marker orientation between the FISH-SA and rheMac2 assembly are boxed. FISH examples supporting the FISH-SA are shown in (**b**) (markers X and Y), and (**c**) (markers N and Q). (**d**) BAC RP11-42F9 (see Table 4) maps within the chromosomal segment of human 4p that, according to the rheMac2 translocated to the tip of chromosome 1 (see Table 1). The FISH experiment did not support the translocation: the BAC mapped to the tip of macaque chromosome 5 (human 4). BACs in green and blue were control markers mapped on MMU5 and MMU1, respectively (see Table 4). (**e**) FISH experiments showed that the BAC RP11-432G15 (internal to the segment that rheMac2 assigns to MMU13 (see Tables 1 and 4) maps on MMU12 (human 2q). BACs in green and blue were used as a control (see Table 4). (**f**) The FISH experiments showed that the BAC RP4-621O15 maps to multiple sites in MMU1. For details see text.

the DNA segment corresponding in humans to chr2:114,076,736–138,830,121 (that is 7.8 megabases exactly downstream the IIp/IIq boundary) on macaque chromosome 13 (IIp). This mapping contradicts the above considerations. Specific FISH experiments confirmed that the segment maps on macaque chromosome 12 (IIq) (Figure 4).

In conclusion, our study demonstrated how assembly uncertainties can be resolved by appropriate FISH experiments illuminated by a careful understanding of evolutionary history.

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