RESEARCH ARTICLE

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Characterization of ancestral chromosome fusion points in the Indian muntjac deer

Abstract Tandem fusion, a rare evolutionary chromosome rearrangement, has occurred extensively in muntjac karyotypic evolution, leading to an extreme fusion karyotype of 6/7 (female/male) chromosomes in the Indian muntjac. These fusion chromosomes contain numerous ancestral chromosomal break and fusion points. Here, we designed a composite polymerase chain reaction (PCR) strategy which recovered DNA fragments that contained telomere and muntjac satellite DNA sequence repeats. Nested PCR confirmed the specificity of the products. Two-color fluorescence in situ hybridization (FISH) with the repetitive sequences obtained and T_2AG_3 telomere probes showed co-localization of satellite and telomere sequences in Indian muntjac chromosomes. Adjacent telomere and muntjac satellite sequences were also seen by fiber FISH. These data lend support to the involvement of telomere and GC-rich satellite DNA sequences during muntjac chromosome fusions.

Introduction

Among mammalian species, diploid chromosome numbers range from a low of 6 in the female Indian muntjac (*Muntiacus muntjak vaginalis*, MMV; Wurster and Benirschke 1970) to peak at $2n=102$ in the red viscacha rat (*Tympanomyctomys barrerae*) (Contreras et al. 1990), whereas most species exhibit chromosome numbers between 36 and 60 (for review see Matthey 1973; Scherthan 2003). The genus *Muntiacus* (small Asian deer) underwent a particularly extreme karyotypic diversification with diploid chromosome numbers ranging from 46 (*M. reveesi*) to 13♀/14♂ (*M. feae*), 8♀/9♂ (*M.*

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crinifrons, *M. gongshanensis*) and 6♀/7♂ (*M. muntjak*) (Wurster and Benirschke 1967; Shi 1983; Soma et al. 1983; Shi and Ma 1988). The common ancestor of all Cervidae is thought to have had a diploid chromosome number of $2n=70$, which may closely resemble the karyotype of *Mazama gouazoubira* and *Hydropotes inermis* (Neitzel 1987). Hsu et al. (1975) proposed that multiple tandem and a few centric fusions shaped the present-day Indian muntjac karyotype, rendering muntjac karyotypic evolution a rare example with tandem fusion being the predominant rearrangement. Since, comparative cytogenetic and molecular cytogenetic studies have supported this hypothesis (e.g., Shi et al. 1980; Neitzel 1987; Lee et al. 1993; Frönicke and Scherthan 1997).

Comparative chromosome painting (Zoo-FISH) among human and different muntjac genomes has delineated regions of conserved segmental homology between Indian muntjac and human, and among Indian muntjac, Chinese muntjac and brown brocket deer complements (Frönicke and Scherthan 1997; Yang et al. 1997). Sites of breakage of chromosomal syntenies in Indian muntjac (MMV) chromosomes most likely represent ancestral fusion points, since these co-localize with interstitial satellite DNAs (Frönicke and Scherthan 1997) and thus might have been involved in the ancestral fusion process (Lin et al. 1991). Further evidence for the fusion hypothesis came with the identification of interstitial $(T_2AG_3)_n$ telomere repeat sequences in Indian muntjac (MMV) chromosomes (Lee et al. 1993; Scherthan 1995; Zou et al. 2002). Interstitial telomeric $(T_2AG_3)_n$ sequences have been found within chromosome arms of many vertebrate species and are considered as the remnants of ancient chromosomal fusions (e.g., Meyne et al. 1990; Vermeesch et al. 1996; Metcalfe et al. 2002; Nanda et al. 2002; Santani et al. 2002).

In *Muntiacus* it has been suggested that the rearrangement of supernumerary centromeres and the particular makeup of repetitive sequences at centromeres and telomeres has contributed to the benign chromosome rearrangements in the evolution of this genus (Brinkley et al. 1984; Elder and Hsu 1988). The concept of tandem

fusion requires numerous ancestral acrocentric chromosomes to fuse head to tail, leading to a centromeric end attached to a previous distal chromosome end. In that scenario, the telomere sequences of the long arm and the pericentromeric sequences must be brought into close proximity and undergo illegitimate exchange (Scherthan 1990). It has been suggested that chromosome fusions may have involved exchange between telomeric repeats and GC-rich pericentromeric Cervidae satellite sequences (Scherthan 1995). Since the latter do not contain T_2AG_3 repeats (Bogenberger et al. 1985; Lin et al. 1991), evidence for the involvement of telomere and centromere sequences in MMV chromosome rearrangements has remained circumstantial (Lee et al. 1993; Li et al. 2000; Zou et al. 2002) with the sequences at the ancestral break and fusion points unknown. To isolate sequences from such fusion points, we designed a composite polymerase chain reaction (PCR) strategy that anchored in telomere and satellite DNA and amplified composite sequences from the muntjac genome. The specificity of the retrieved sequences was confirmed by nested PCR. These fragments also co-localized with interstitial telomere and satellite DNA sequences in MMV chromosome arms, suggesting that they represent ancestral chromosome fusion sites.

Materials and methods

Chromosome and chromatin fiber preparation

Indian muntjac fibroblasts (Scherthan 1990) were grown in Eagle's minimal essential medium (Bio Whittaker) enriched with 1% L-glutamine, 1% penicillin-streptomycin and 10% fetal bovine serum (Bio Whittaker). For metaphase spreads, cells were treated with a final concentration of 10 μg/ml Colcemid (Biochrome) 2 h prior to harvesting. After harvesting cells by 1% EDTA, PBS treatment they were incubated in 50 mM KCl solution for 15 min at 37°C and fixed in 3:1 methanol:acetic acid for 30 min on ice. After a minimum of five washing steps with ice-cold fixative, the suspension was dropped onto ethanol-cleaned slides. Preparations were exposed to warm water vapor for 10 s, air-dried and stored at −20°C.

For chromatin fibers, cells were pelleted and incubated in 50 mM KCl solution for 20–30 min at 37°C and then mixed with 0.1 M NaOH on a clean glass slide. Chromatin fibers were produced by pulling the lysed cell suspension with a coverslip slowly over the slide. Finally, these preparations were air-dried and fixed in ice-cold 3:1 methanol:acetic acid for 10 min.

Polymerase chain reaction

DNA was isolated from Indian muntjac fibroblasts with the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. Isolated DNA was shortened to a fragment size of 200–1000 bp by sonification (Branson). Primers used in PCR were: TeloG (5′-GGTTAGGGTTAGGGTTAGGG-3′); TeloC (5′-CCTAACCCTAACCCTAACC-3′); SatIA (5'-CCCCGCCTAACTCG); Munt2 (5′-TCGAGAGGAATCCTGAG-3′); and nested breakpoint-spanning primers TGS400 fw (5′-GTTAGGGTGGAGGCCGCAA-3′); TGM225 fw (5′-GTTAGGGCATCTCGGGGTC-3′); TGM and S rv (5′-CCTGGCTTCAAATCAAGAGG-3′); TCS165 fw (5′-CTAACCCTTTGACTGTGTGG-3′); TCS165 rv (5′-CAGGTCTCGCCGCAACTAG-3′). Satellite (Sat) II primers were used according

to Li et al. (2000, 2002): SatII-fw (5′-GAGCTGCCTGACAGACTCG-3′); SatII-rv (5′-CAGAGCCGACCTAGGATCAC-3′); SatII-modified-fw (5′-GACTGATTTCCTGGGTTAAGAG-3′); and SatII-modified-rv (5′-CACACAGAATGCTAGGAAATCC-3′). The PCR was carried out in a 50 μl reaction volume using 100 ng template DNA, 200 μM of each dNTP, 1×PCR-buffer, 1.5 mM MgCl$_2$, 0.2 μM of each primer and 2.5 U Taq Polymerase (Qiagen). A negative control (without template DNA) was always included.

Touch-down PCR was performed with the following cycling conditions: initial denaturation (3 min at 94°C), 10 touch-down cycles (30 s 94°C, 30 s 57°C with temperature decrease of 0.5°C per cycle to 52°C, 1 min at 72°C), thereafter 30 cycles (30 s 94°C, 30 s 52°C, 1 min 72°C) and a final extension step (10 min at 72°C). Conditions for nested PCR were as above except for 10 touch-down cycles (30 s 65°C and temperature decrease of 0.5°C per cycle to final 60°C) followed by 30 cycles (30 s 94°C, 30 s 60°C, 1 min 72°C) and a final extension step (10 min at 72°C).

Cloning and DNA sequence analysis

The PCR products of interest were purified from a 1% agarose gel, ligated into the T/A overhang vector pGEM-T easy (Promega) and transformed in competent *Escherichia coli* XL1-Blue cells. Plasmids were purified from randomly picked clones and checked for the presence of the insert by restriction analysis. Cloned inserts were sequenced from both sides using the Big Dye Terminator kit (PE-Biosystems) and an ABI Prism sequencer (Applied Biosystems). The complete DNA sequences of the Telo-Sat clones were deposited in the GenBank database (Accession numbers: AY322158, AY322159, AY322160).

Sequence analysis was carried out using BLASTn at NCBI (http://www.ncbi.nlm.nih.gov) and RepeatMasker at Baylor HGSC (http://searchlauncher.bcm.tmc.edu). ClustalW (http://www.ebi.ac.uk/clustalw) was used for sequence comparison.

Fluorescence in situ hybridization

Plasmids containing PCR products were labeled with the Digoxigenin Nick Translation kit (Roche) according to the manufacturer's instructions. Metaphase preparations were hydrated in PBS, pH 7.4 for 5 min and then fixed in 3.7% formaldehyde, PBS for 5 min at room temperature and washed three times in PBS, 0.1% glycine. Both metaphase and DNA fiber preparations were treated for 15 min with 1 M sodium thiocyanate at 90°C; denaturation was performed in 70% formamide, 2×SSC, pH 7.1 for 2 min at 68°C (metaphase spreads) and for 4 min at 72°C (fiber preparations). Hybridization mixture (50% formamide, 10% dextran sulfate in 2×SSC, containing the labeled probes (50 ng/μl) and E. coli carrier DNA (1 μg/μl), was denatured at 95°C for 5 min and applied to the slides. Hybridization was allowed to proceed for 96 h in a wet chamber at 37°C. Telomeres on metaphase spreads were detected with a directly fluorescein isothiocyanate (FITC)-labeled PNA probe (Dako) prior to FISH with the PCR products, whereas telomere sequences on fiber preparations were detected with a biotinylated 42-mer telomere probe (Scherthan 2002). For detection of fusion point PCR products and telomere sequences, we first denatured the slides in the presence of a fluorescein-labeled $(C_3TA_2)_3$ telomere PNA probe (Dako) followed by 4 h of hybridization to saturate telomere sequences. Then, the coverslip was lifted and the denatured composite satellite PCR DNA probe was applied and sealed again under a larger coverslip followed by hybridization overnight.

Stringent washing was performed twice for 5 min in 0.05×SSC at 42°C. Hybrid molecules were visualized by rhodamine-conjugated anti-digoxigenin Fab fragments (Roche) and/or by Extravidin-FITC (Sigma) for biotin molecules (for details see Scherthan 2002). Slides were embedded in antifade solution (Vector Laboratories) supplemented with 4′,6-diamidino-2-phenylindole (DAPI) (0.5 μg/ml) and Actinomycin D (10 μg/ml).

Microscopy

Fluorescent signals were captured on a Zeiss Axioskop fluorescence microscope equipped with a cooled CCD camera (Hamamatsu) and with single band pass filters for excitation of DAPI, FITC and rhodamine. Images were enhanced and analyzed using the ISIS software (MetaSystems).

Results

Composite Telo-Sat PCR in the MMV genome

It has been predicted that chromosome fusions in muntjac evolution involved breaks and repair between satellite DNA and telomere sequences (Scherthan 1995). If so this should have led to direct fusion of telomere and satellite sequences. To test for the presence and to isolate such fusion junctions we designed PCR primers for Indian muntjac DNA satellite IA (Bogenberger et al. 1982) (primer SatIA) and Chinese muntjac DNA satellite I (C5; Lin et al. 1991) (primer Munt2) and combined each of these satellite DNA primers with one telomere-specific primer $(T_2AG_3)_3$ (TeloG) or $(C_3TA_2)_3$ (TeloC). These primer combinations were assumed to amplify an ancestral fusion point when satellite and telomere DNA sequences were fused and sufficiently close. In order to restrict PCR amplification to closely spaced sequences, muntjac genomic template DNA was sonified to a size of about 200–1000 bp prior to PCR. First, we performed a single primer reaction on sonified MMV template DNA with all primers. All reactions were negative except for one prominent band of about 800 bp obtained with primer SatIA (Fig. 1A). This indicates that the SatIA repeats are present in inverse orientation in the Indian muntjac genome, which is likely the case at the large X centromere, which harbors SatIA sequences (Bogenberger et al. 1987) and because the MMV X has been created by a Robertsonian fusion between the actual X and an autosomal long arm. The combination of C- and G-rich telomere primers resulted in a smear, which agrees with earlier data (Ijdo et al. 1991). Single telomere primers failed to amplify DNA sequences in the MMV genome, which suggests a dearth of head-to-head fusions of terminal telomere repeats.

Next we combined telomere and satellite primers and performed PCR on MMV template DNA. This composite PCR repeatedly revealed conspicuous PCR bands for all heterologous primer pairs in the range of 165 to 500 bp (Fig. 1A). The primer combination TeloG/SatIA produced a 400 bp product (designated TGS400), whereas TeloG/Munt2 resulted in two distinct bands at 225 bp (TGM225) and at 451 bp (TGM451). TeloC/SatIA resulted in a 165 bp and a 349 bp product (TCS165 and TCS349), while TeloC/Munt2 generated a 431 bp product (TCM431) (Fig. 1A; Table 1). All reactions were repeated several times with different sheared MMV DNAs and always revealed similar reaction products. A number of composite PCR products (Fig. 1) were then isolated, cloned and subjected to sequence analysis. Given the large number of fusions in the course of muntjac evolution we expect that we have isolated only a subset of fusion point sequences.
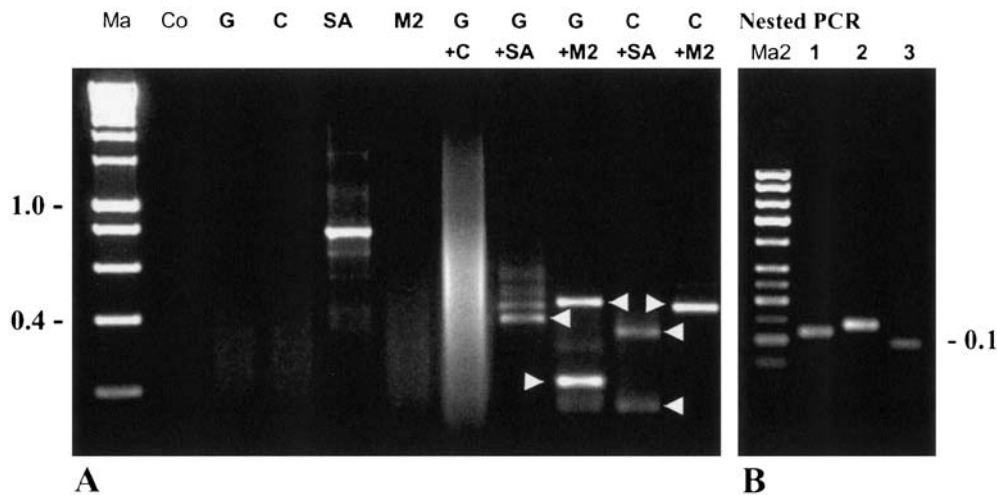

Fig. 1 A Composite polymerase chain reaction (PCR) using different combinations of telomere (TeloG *G*; TeloC *C*) and satellite (SatIA *SA*; Munt2 *M2*) primers. Single primer reactions generated one prominent band at about 800 bp with the SatIA primer (*SA*). The reaction with TeloG and TeloC telomere primers produced an expected smear (*G+C*), whereas the different combinations of one telomere with one satellite primer resulted in several prominent bands. (*Co* negative control, *Ma* marker) *Arrowheads* mark products: G+SA, TGS (400 bp); G+M2, TGM (451 bp); G+M2, TGM (225 bp); C+SA, TCS (349 bp); C+SA, TCS (165 bp); C+M2, TCM (431 bp). These bands were excised and used for sub-cloning and sequence analysis. Sizes of marker bands are indicated in kilobases. **B** Nested PCR using fusion point-spanning primers (see Materials and methods) and sheared genomic template DNA confirm specificity of the primary PCRs. Nested primer combinations reveal CPR fragments corresponding to: TGS400 (*lane 1*); TGM225 (*lane 2*); TCS165 (*lane 3*)

Table 1 Composite primer polymerase chain reaction (PCR) and similarities of composite primer products to sequences in the databases

PCR product no.	Composite primer combination	Length of PCR product (bp)	Most similar sequences to the non-telomeric part of the PCR product (Genbank Accession no.)
TGS400	TeloG + SatIA	400	97% (211 bp), Muntjak satellite IA sequence (X02323.1)
			94% (177 bp), *Muntiacus reevesi* repetitive DNA sequence (X56823.1)
			82% (343 bp), *Cervus elaphus canadensis* centromeric satellite DNA (U53516.1)
TGM451	TeloG + Munt2	451	–
TGM225	TeloG + Munt2	225	93% (132 bp), *M. reevesi* repetitive DNA sequence (X56823.1)
			90% (155 bp), Muntjak satellite IA sequence (X02323.1)
			90% (63 bp), *Alces alces* centromeric satellite DNA (U53518.1)
TCS349	TeloC + SatIA	349	93% (114 bp), *Bos taurus* clone RP42-354B6 (AC091660.2)
			94% (110 bp), *B. taurus* clone rp42-158g13 (AC105306.13)
			94% (118 bp), *B. taurus* butyrophilin gene AF005497.1
TCS165	TeloC + SatIA	165	92% (116 bp), *C. elaphus canadensis centromeric satellite DNA (U53516.1)*
			90% (122 bp), *Odocoileus hemionus* centromeric satellite DNA (U55814.1)
			89% (117 bp), Muntjak satellite IA sequence (X02323.1)
TCM431	TeloC + Munt2	431	90% (1449 bp), *B. taurus* kappa casein (CSN3) gene, intron 2 (AY185364.1)
			91% (132 bp), *B. taurus* clone rp42-513g13 (AC107065.4)
			90% (133 bp), *B. taurus* clone RP42-518P7 (AC129959.6)

The names and Accession numbers of the most similar sequences to each PCR-product are given according to BLASTn at NCBI. Similarity (%) refers to the most homologous sequence string of the PCR-products. No sequence similarity was found for TGM451

Sequences of putative chromosome fusion sites

Sequence analysis revealed that three out of six cloned PCR products isolated with one telomere and one satellite primer, namely TGS400, TGM225 and TCS165 (Accession numbers AY322158, AY322159, AY322160), contained several (T_2AG_3) repeats that were directly fused to muntjac satellite DNA sequences (Fig. 2). These satellite sequences shared highest similarity with the MMV satellite IA sequence, the *M. reevesi* C5 satellite DNA, and satellite sequences from other deer species (Table 1).

When we performed ClustalW alignment with the PCR products obtained with the G-rich telomere primer and either of the satellite primers, it became apparent that the T_2AG_3 repeats in the TGM and TGC products were fused to the same strand of MMV SatIA DNA, but at different nucleotide positions (Fig. 2). The sequence of the PCR product TGM451, also obtained with the G-rich telomere and the Munt2 primer, was not similar to any DNA sequence currently stored in the databases.

In contrast, one of the composite PCR products obtained with the C-rich telomere primer (TeloC) and one satellite primer showed telomere repeats adjacent to cervid- and muntjac-specific satellite DNA (Fig. 2). However, the telomere-satellite sequence fusion likely occurred between Watson and Crick strands as compared with the TGS400 and TGM225 sequences. On the other hand, PCR products TCS349 and TCM431 displayed a high sequence similarity to non-coding sequences from various bovine DNA clones in the database: a repetitive sequence string similar to a human LINE-like repeat in TCM431 and a short sequence string similar to human tRNA in TCS349 (Table 1).

In order to exclude the possibility that primer artifacts may have caused the direct proximity of telomere and satellite sequences in the composite PCR products, we designed nested primers that span the fusion point between telomere and satellite DNA sequences. When this breakpoint-spanning nested PCR was performed with sheared genomic Indian muntjac DNA as template it generated products of the expected size, thereby corroborating the idea that the telomere-satellite sequence junctions isolated from the Indian muntjac genome represent authentic fusion sites (Fig. 1B).
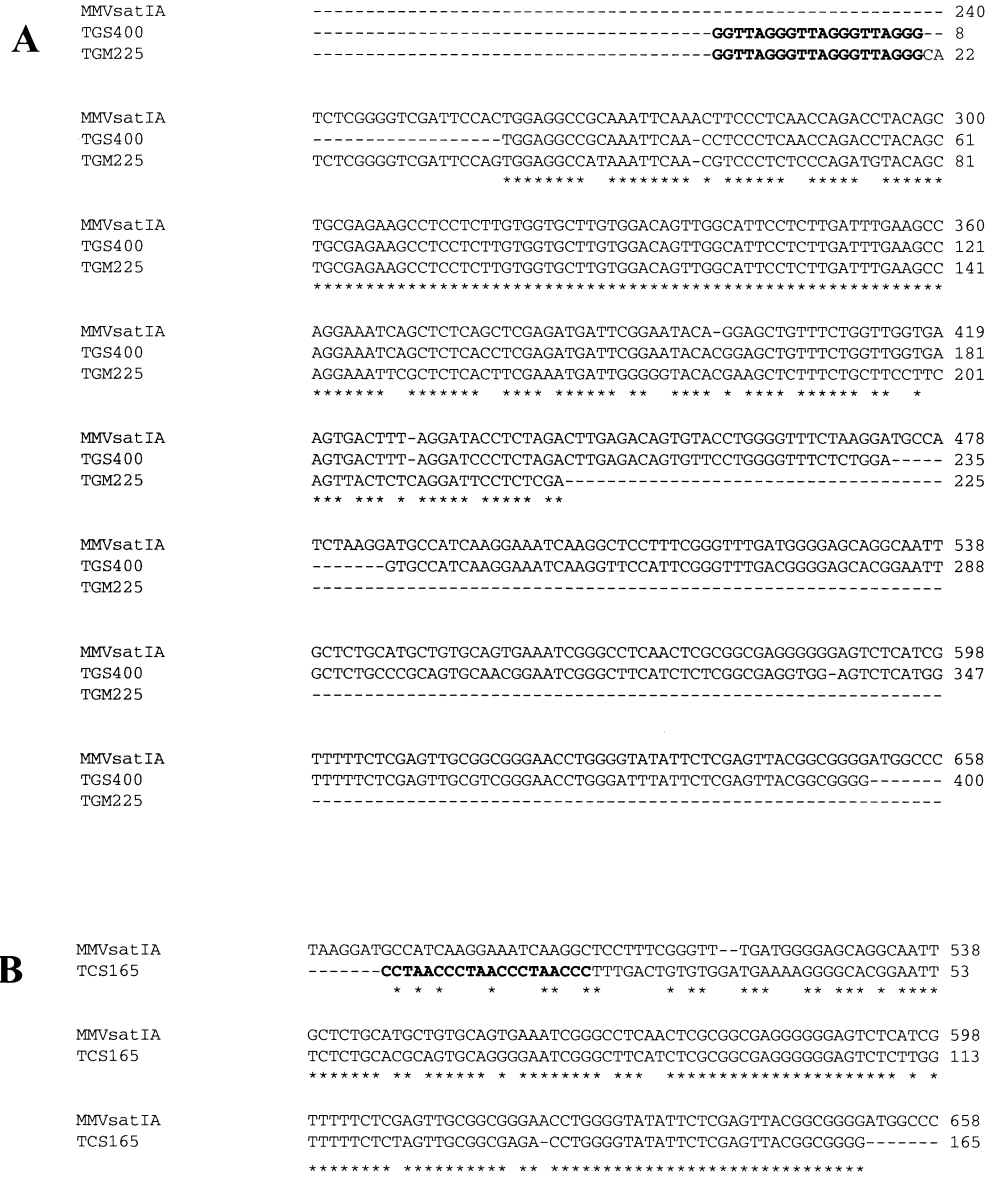
Altogether, the above results show that the G-rich strand of telomere sequences was often fused with satellite sequences that preferentially reside in pericentromeres, as is the case for the C5 sequence in the more ancestral-like Chinese muntjac karyotype (Lin et al. 1991).

Recently, it has been proposed that the Indian muntjac chromosome fusions led to the deletion of some SatII repetitive sequences during the fusion process (Li et al. 2000). To test for the potential joining of SatII sequences to telomere repeats, we used satellite II primers (Li et al. 2000, 2002) in combination with TeloG and TeloC on MMV template DNA. These composite PCRs produced several bands but sequence analysis of the fragments amplified between the telomere and SatII primer binding sites failed to reveal any similarity to sequences in the databases (data not shown).

Composite PCR products map to interstitial sites in MMV chromosomes

To determine the physical positioning of the site from which the composite PCR products originated, we performed single-color FISH with labeled PCR products. The FISH patterns obtained with the three TeloG-Sat PCR products, TGS400, TGM225 and TCS165, generated interstitial signals on MMV chromosomes. Signals of

Fig. 2 Sequence comparison of PCR products TGS400, TGM225, and TCS165 (Accession numbers AY322158, AY322159, AY322160, respectively) with Indian muntjac satellite IA sequence (*MMVsatIA*, Accession number X02323, Bogenberger et al. 1985). The telomere sequence string at one end is marked in *bold*. **A** The sequences TGS400 and TGM225, picked by satellite DNA and the G-rich telomere primer, show high homology to part of MMV satellite IA DNA. **B** Sequence TCS165, generated with the C-rich telomere primer and a SatIA primer, aligns with part of the reverse and complementary strand of MMV satellite IA

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A   MMVsatIA   ---------------------------------------------------------- 240
    TGS400     -----------------------------------GGTTAGGGTTAGGGTTAGGG-- 8
    TGM225     -----------------------------------GGTTAGGGTTAGGGTTAGGGCA 22

    MMVsatIA   TCTCGGGGTCGATTCCACTGGAGGCCGCAAATTCAAACTTCCCTCAACCAGACCTACAGC 300
    TGS400     -----------------TGGAGGCCGCAAATTCAA-CCTCCCTCAACCAGACCTACAGC 61
    TGM225     TCTCGGGGTCGATTCCAGTGGAGGCCATAAATTCAA-CGTCCCTCTCCCAGATGTACAGC 81
                                ********  ******** * ****** ***** ******

    MMVsatIA   TGCGAGAAGCCTCCTCTTGTGGTGCTTGTGGACAGTTGGCATTCCTCTTGATTTGAAGCC 360
    TGS400     TGCGAGAAGCCTCCTCTTGTGGTGCTTGTGGACAGTTGGCATTCCTCTTGATTTGAAGCC 121
    TGM225     TGCGAGAAGCCTCCTCTTGTGGTGCTTGTGGACAGTTGGCATTCCTCTTGATTTGAAGCC 141
                ***********************************************************

    MMVsatIA   AGGAAATCAGCTCTCAGCTCGAGATGATTCGGAATACA-GGAGCTGTTTCTGGTTGGTGA 419
    TGS400     AGGAAATCAGCTCTCGACCTCGAGATGATTCGGAATACACGGAGCTGTTTCTGGTTGGTGA 181
    TGM225     AGGAAATTCGCTCTCACTTCGAAATGATTGGGGGGTACACGAAGCTCTTTCTGCTTCCTTC 201
                ******* ******* **** ****** **  **** * **** ****** **   *

    MMVsatIA   AGTGACTTT-AGGATACCTCTAGACTTGAGACAGTGTACCTGGGGTTTCTAAGGATGCCA 478
    TGS400     AGTGACTTT-AGGATCCCTCTAGACTTGAGACAGTGTTCCTGGGGTTTCTCTGGA----- 235
    TGM225     AGTTACTCTCAGGATTCCTCTCGA---------------------------------- 225
                *** *** * ***** ***** **

    MMVsatIA   TCTAAGGATGCCATCAAGGAAATCAAGGCTCCTTTCGGGTTTGATGGGGAGCAGGCAATT 538
    TGS400     -------GTGCCATCAAGGAAATCAAGGTTCCATTCGGGTTTGACGGGGAGCACGGAATT 288
    TGM225     ----------------------------------------------------------

    MMVsatIA   GCTCTGCATGCTGTGCAGTGAAATCGGGCCTCAACTCGCCGGCGAGGGGGGAGTCTCATCG 598
    TGS400     GCTCTGCCCGCAGTGCAACGGAATCGGGCTTCATCTCTCGGCGAGGTGG-AGTCTCATGG 347
    TGM225     ----------------------------------------------------------

    MMVsatIA   TTTTTCTCGAGTTGCGGCGGGAACCTGGGGTATATTCTCGAGTTACGGCGGGGATGGCCC 658
    TGS400     TTTTTCTCGAGTTGCGTCGGGAACCTGGGATTTATTCTCGAGTTACGGCGGGG------- 400
    TGM225     ----------------------------------------------------------
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B   MMVsatIA   TAAGGATGCCATCAAGGAAATCAAGGCTCCTTTCGGGTT--TGATGGGGAGCAGGCAATT 538
    TCS165     -------CCTAACCCTAACCCTAACCCTTTGACTGTGTGGATGAAAAGGGGCACGGAATT 53
                    * * *     *    ** **         * **   ***   ** *** * ****

    MMVsatIA   GCTCTGCATGCTGTGCAGTGAAATCGGGCCTCAACTCGCCGGCGAGGGGGGAGTCTCATCG 598
    TCS165     TCTCTGCACGCAGTGCAGGGGAATCGGGCTTCATCTCGCCGGCGAGGGGGGAGTCTCTTGG 113
                ******* ** ****** * ******** ***    ********************* * *

    MMVsatIA   TTTTTCTCGAGTTGCGGCGGGAACCTGGGGTATATTCTCGAGTTACGGCGGGGATGGCCC 658
    TCS165     TTTTTCTCTAGTTGCGGCGAGA-CCTGGGGTATATTCTCGAGTTACGGCGGGG------- 165
                ******** ********** ** ****************************
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strong intensity were obtained at the euchromatin/heterochromatin boundary of the large X centromere, intermediate signals at the centromeres of chromosome 1 and 2, and a total of 25–27 weak signals at interstitial sites (Fig. 3a), which agrees with the FISH pattern reported for the C5 Chinese muntjac satellite probe (Lee et al. 1993; Frönicke and Scherthan 1997). The results also show that FISH with these composite satellite PCR products predominantly labels satellite loci on MMV chromosomes.

In contrast, FISH of PCR products TGM451 and TCM431 failed to reveal signals, while TCS349 painted the entire euchromatin of MMV chromosomes (Fig. 3b), a pattern that has been noted for a dispersed repeat previously isolated from a MMV microdissection library (H.S., unpublished). Finally, FISH experiments with the composite Telo/SatII PCR products (see above) as probes failed to reveal signals on MMV chromosomes (not shown), suggesting that direct fusions between MMV SatII and telomere repeats are rare events, if they occur at all.

Fiber FISH

Next we attempted to gain further information about the physical proximity of telomere and satellite DNAs at a high resolution in the muntjac genome by fiber FISH. Since the composite TeloG/satellite PCR products largely delineate interstitial satellite sequences in MMV chromosomes (see above), we discriminated between the satellite and telomere sequences by two-color fiber FISH using a biotinylated telomere probe and the differentially labeled satellite-like PCR product TGS400. This approach revealed several differentially colored FISH signal tracks on chromatin fibers where a short telomere signal array
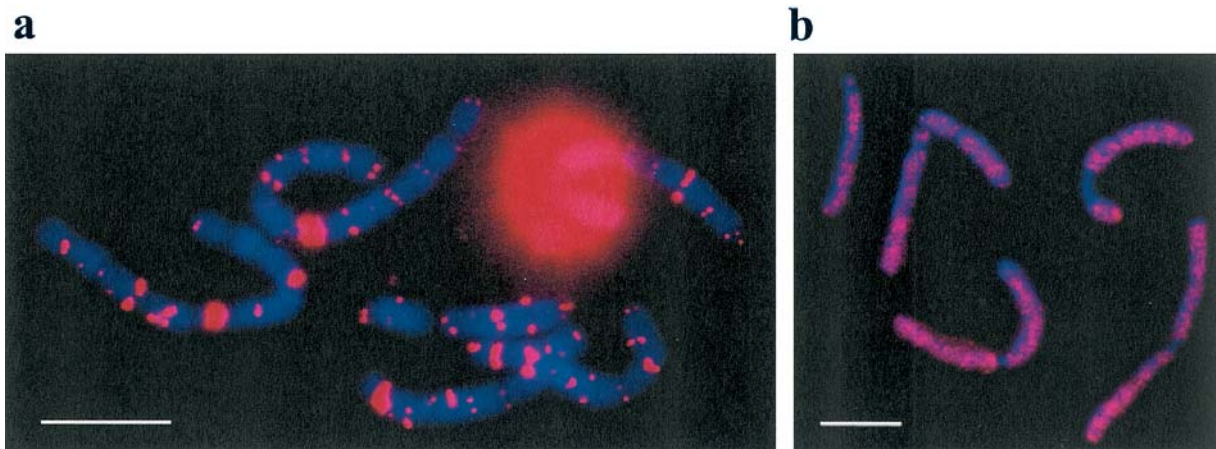
Fig. 3 a Fluorescence in situ hybridization (FISH) of the satellite-like PCR product TGS400 (*red signals*) to metaphase spreads of male Indian muntjac (*Muntiacus muntjak vaginalis*, MMV) fibroblasts produces numerous interstitial signals. Very strong signals are seen at the euchromatin/heterochromatin boundary of the X centromere (*huge red blur*), strong signals at the centromeres of chromosome 1 and 2, while numerous interstitial signals are smaller. The signal distribution is similar to that obtained with the *M. reveesi* satellite DNA C5 probe (Lin et al. 1991). **b** The TCS349 product revealed FISH signals dispersed throughout the euchromatic MMV chromosome arms. *Bars* represent 10 μm
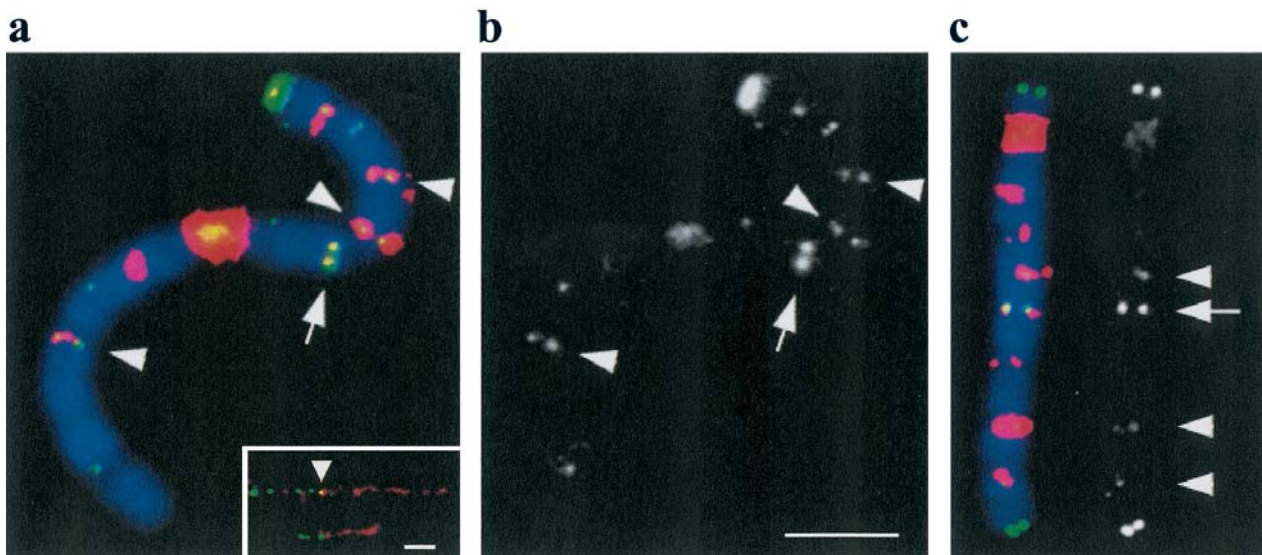
Fig. 4 Two-color FISH of T₂AG₃ telomere repeats (*green* in the merged images and *gray* in the gray channel images) and satellite-like TGS400 sequence (*red* in the merged images). **a** MMV chromosome 1 showing several interstitial T_2AG_3 repeat signals (*arrowheads*) and one strong interstitial telomere repeat signal (*arrow*) that colocalize with the TGS400 satellite-like PCR sequences. Terminal telomere signals are strong at MMV 1p ter and faint at MMV1q ter. **b** *Gray* channel corresponding to **a** showing telomere repeat signals only. **c** MMV 2 showing terminal telomere signals and three weak (*arrowheads*) and one strong interstitial telomere repeat signal (*arrow*) that colocalize with the TGS400 sequence. The *gray* channel showing telomere repeat signals only is seen to the *right*. *Bar* in **b** represents 5 μm and applies to **a–c**. *Inset* Fiber FISH with the TGS400 satellite-like PCR probe highlighting proximity of pericentric satellite DNA (*red*) and telomere repeats (*green*) on extended MMV chromatin fibers. Telomere and TGS400 satellite signal strings are juxtaposed. One signal spot in the upper fiber shows a mixed color (*arrowhead*). The lower fiber displays adjacent telomere and satellite signal spots. *Bar* represents 2 μm

directly neighbored a satellite array in a bead-on-a-string fashion; occasionally we observed beads that displayed a mixed color, suggesting that both telomere and satellite DNA sequences may be present in such a spot (Fig. 4a, inset). Other two-color signal arrays contained telomere signals within a string of satellite signals (not shown), which may result form telomere sequences embedded, e.g., in the large X centromere (Scherthan 1990). Given the resolution limit of fiber FISH being in the 1 kb range (Florijn et al. 1995), we expect that we have missed very short telomere sequences and thus a number of potential junction fragments by this approach.

Colocalization of T_2AG_3 and satellite DNA sequences in MMV chromosomes

Since the patterns obtained with fiber FISH agree with a juxtaposition of telomere and satellite DNA repeats but extended chromatin fibers do not allow these sites to be physically mapped to individual chromosome regions, we next mapped telomere and satellite PCR products by two-color FISH on metaphase chromosomes. To identify telomere sequences unequivocally we first stained T_2AG_3 repeats with a fluorescein-labeled PNA probe and, after 4 h hybridization, added the differentially labeled composite satellite TGS400 PCR sequence probe for hybridization to satellite sequences.

These two-color FISH experiments with Tel/Sat composite PCR sequence probe and the differentially labeled PNA telomere sequence probe revealed several interstitial T_2AG_3-specific signals that co-localized with interstitial satellite sequences highlighted by the Tel/Sat composite probe (Fig. 4), revealing the juxtapositioning of telomere and fusion-specific muntjac Telo-Sat PCR products in MMV chromosomes.

Discussion

Tandem fusion represents the major type of rearrangement that led to the present-day karyotype of the Indian muntjac. Recent phylogenetic data suggest that karyotypic evolution occurred linearly, leading from the putative ancestral number (2n=70) over a Chinese muntjac-like (2n=46) to a Fea's muntjac-like ancestral complement of 2n=13/14 (Wang and Lan 2000), while the chromosomal reduction from the Fea's muntjac-like complement to the Black and Gongshan muntjac (2n=8/9) and to the Indian muntjac complements (2n=6/7) most likely occurred independently (Wang and Lan 2000). According to the latter authors, the first common ancestor of the genus *Muntiacus* lived 1.9–3.7 millions of years ago, which represents an extremely short period for such a drastic chromosome reduction.

According to Wichman et al. (1991), radically reorganized karyotypes will retain molecular evidence of the mechanisms that have shaped the present-day karyomorphs. In agreement with the perception that interstitial telomeric sites often represent remnants of ancient fusion sites in mammals (e.g., Meyne et al. 1990; Vermeesch et al. 1996; Ruiz-Herrera et al. 2002), several interstitial telomeric sequences have been localized in Indian muntjac chromosomes (Lee et al. 1993; Scherthan 1995) and map to one-third of all fragile sites in this complement (Zou et al. 2002). In hominoids fragile sites also correlate with a fraction of putative ancestral fusion sites (see Ruiz-Herrera et al. 2002). In this respect it is interesting to note that the extant human chromosome 2 displays head-to-head telomere sequence remnants at the fusion point (Ijdo et al. 1991). However, other types of mechanisms also have the potential to seed interstitial telomere repeats (see Azzalin et al. 2001).

In the Indian muntjac genome, PCR with a single telomere primer failed to generate a specific product, which makes direct head-to-head telomere fusions an unlikely event in MMV chromosome evolution. This supports other types of rearrangements, including a scenario that invokes distal telomere/satellite sequence exchange. Composite PCR with TeloC and Sat primers revealed sequences with homology to bovine sequences in the database. Given that these TeloC/Sat PCR products generated dispersed FISH patterns on MMV chromosomes it seems likely that these sequences may have been created by retroposition-associated events.

In MMV chromosomes, remnants of the Chinese muntjac C5 satellite I DNA as well as MMV SatII are present at up to 27 interstitial sites (Lin et al. 1991; Li et al. 2000) that co-localize with ancestral break/fusion points as defined by boundaries of conserved syntenic segments (Frönicke and Scherthan 1997). Telo-Sat composite PCR generated products that contained both telomere and Indian muntjac satellite I repeats, while Telo-Sat junctions were not retrieved from *M. reversi* DNA (unpublished results). These composite MMV-specific PCR products stained the interstitial satellite loci in MMV chromosomes and colocalized with telomere sequences as detected by two-color FISH experiments with a T_2AG_3 PNA probe within MMV chromosome arms. Additionally, fiber FISH experiments revealed short telomere signal strings in the vicinity of satellite signals, which is consistent with the sequences of our telomere/satellite PCR products.

Previously, Li et al. (2000) suggested that most ancestral chromosome fusion sites result from breaks in satellite II, telomere or other sequences. Since we repeatedly picked satellite I/telomere junctions by compositie PCR but failed to capture direct telomere/satellite II successions, it seems more likely that double-strand breakage and repair involved satellite I and telomere sequences. Deletion of the centromere-associated satellite II (Vafa et al. 1999) may have contributed to centromere inactivation during generation of the MMV fusion chromosomes. Furthermore, composite PCR products that contained telomere and non-satellite or unknown sequences may stem from other types of rearrangements and/or reflect different orientations relative to a centromere/telomere axis.

Altogether, three different approaches, namely composite PCR/nested PCR, fiber FISH and metaphase FISH disclosed juxtaposition of telomere and satellite I sequences in the MMV genome. The large number of interstitial satellite sites in the MMV complement prevented the mapping of a particular fusion point sequence to a single site. Thus, future work will have to isolate fusion point-containing cosmids. It is tempting to speculate that the sequences obtained by our composite PCR approach have been created by illegitimate recombination between telomeric and satellite DNA, an assumption that will be explored in future in vitro analysis.

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