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Origin of an apparent B chromosome by mutation, chromosome fragmentation and specific DNA sequence amplification

Received: 18 June 2002 / Revised: 30 July 2002 / Accepted: 19 August 2002 / Published online: 2 October 2002
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Abstract The present study documents the de novo origin of an apparent B chromosome in *Plantago lagopus*. The origin was associated with mutation (aneuploidy), chromosome fragmentation, specific DNA sequence amplification, addition of telomeric repeats, and centromeric misdivision. It originated in the progeny of trisome 2, from the excision of 5S rDNA and 18S, 5.8S, 25S rDNA sequences located on chromosome 2, and within a few generations acquired many characteristics of an apparent B chromosome. The B chromosome has preferential transmission through the male (41%, $P < 0.025$) and female gametes (42%, $P < 0.01$) but does not affect plant phenotype. The B chromosome is completely heterochromatic, has a functional centromere and does not pair at meiosis with any A chromosomes of the standard complement. Fluorescence in situ hybridization analysis showed that it arose from massive amplification of 5S rDNA sequences, has 18S, 5.8S, 25S rDNA sequences at the ends of both arms and telomeric repeats at both termini. Ag-NOR-banding and determination of the maximum number of nucleoli in interphase cells indicate that the nucleolar organizer regions at the ends of both arms of the B chromosome are active in organizing nucleoli. RNA blot analysis showed that the 5S rDNA sequences are not transcribed. To our knowledge, this is the first report that fully documents one of the mechanisms by which B chromosomes may arise in nature.

Edited by: P. Moens

Accession numbers: pPov1, AF464933; *Plantago lagopus* 5S rRNA, AF464934

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Introduction

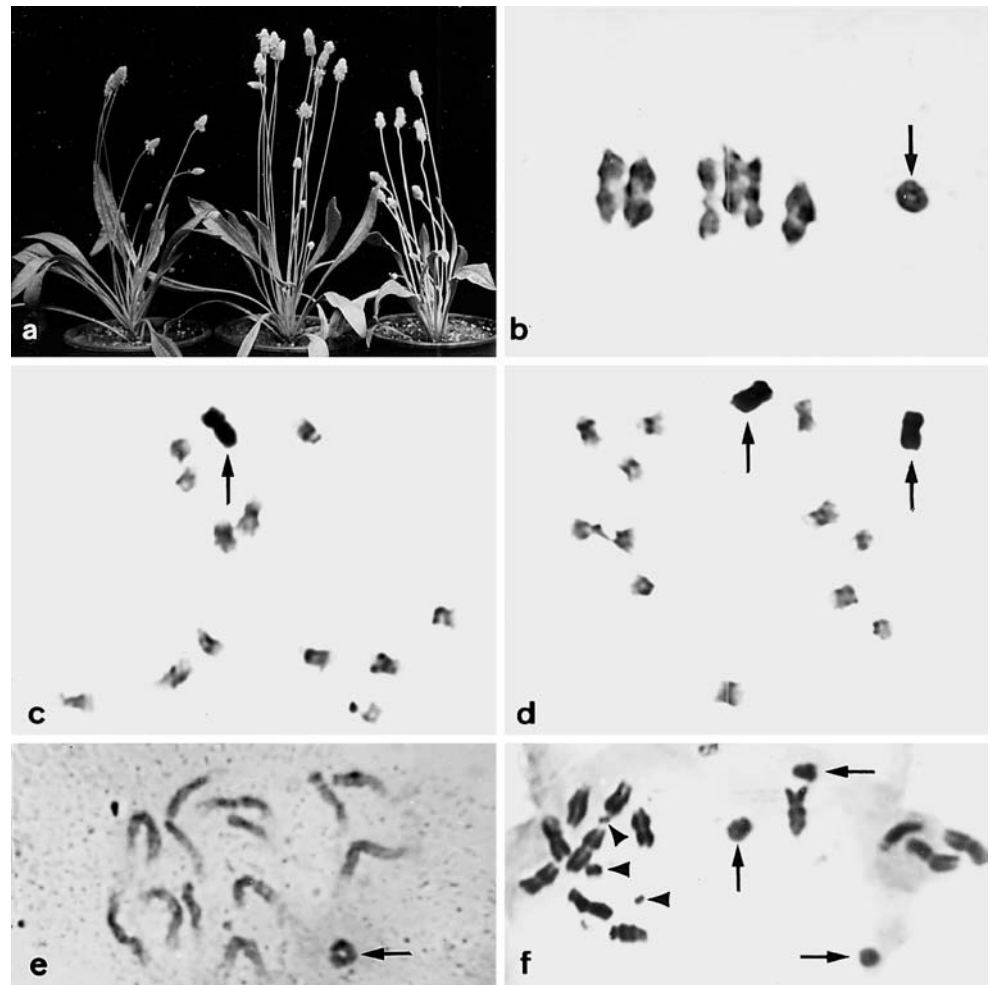
B chromosomes are dispensable supernumerary chromosome additions to the standard A chromosome complement of a species that may or may not be present in certain tissues, individuals, or populations. B chromosomes do not pair and recombine with any of the A chromosomes at meiosis, are often heterochromatic, and usually do not contain major genes except for ribosomal DNA (rDNA) sequences that have been mapped on many plant and animal B chromosomes (Jones and Rees 1982; Jones 1995). Although B chromosomes are usually detrimental to their carrier, they are found in many species. The reason for this paradox is that B chromosomes have developed drive mechanisms that ensure their persistence in populations (Jones 1991).

Although a lot of work has been conducted on the structure, occurrence, sequence analysis, meiotic behavior and transmission of B chromosomes, their origin is still an enigma (Jones 1995; Langdon et al. 2000). It is widely accepted that B chromosomes originate from standard A chromosomes. However, there is also evidence suggesting that B chromosomes are derived from alien chromosomes introgressed from related species by interspecific hybridization (Camancho et al. 2000). Irrespective of the intra- or interspecific origin of B chromosomes, to quote Jones (1995), “what we don’t catch... is the really rare event where a fragment is born which simultaneously seizes immortality”. We were able to observe such a rare event in the plant genus *Plantago* and document here one of the mechanisms by which a B chromosome may arise in nature.

Plantago lagopus L. ($2n=2x=12$) is one of the 280 species comprising the genus *Plantago* of the family Plantaginaceae. A small herb about 30 cm tall, *P. lagopus* grows as a weed in the Mediterranean region. *P. lagopus* is partly outbreeding and related to *Plantago ovata* Forsk., which is grown as a medical crop in India.

In 1982, a spontaneous trisomic plant ($2n=2x=13$, Triplo 4) of *P. lagopus* was recovered in an experimental population at Jammu, India (Sharma and Koul 1984). The

Fig. 1 Plant morphology and chromosome structure of *Plantago lagopus*: **a** morphology of plants (from left to right) with $2n=12$, $2n=12+i$, and $2n=12+2i$ chromosomes; **b** a pollen mother cell at meiotic metaphase I of a plant with $2n=12+i$ chromosome paired as six ring bivalents plus a ring univalent (arrow); **c, d** C-banded mitotic metaphase chromosomes of plants with $2n=12+i$ (**c**) and $2n=12+2i$ (**d**) (arrows indicate the heterochromatic isochromosomes); **e, f** mitotic prometaphase (**e**) and metaphase chromosomes (**f**) of a plant with $2n=12+r$ chromosomes recovered in the progeny of the cross of Triplo 2 and euploid plants. Note that the metaphase shown in **f** is from the same individual but has $2n=12+3r$ (arrows) +3dm (arrowheads) chromosomes



origin of this trisomic plant was attributed to nondisjunction, a feature observed in about 3% of the pollen mother cells of normal euploid plants. Subsequent crossing of Triplo 4 with euploid plants revealed the tremendous plasticity of the genome, and plants with 13 to 28 chromosomes were recovered over a period of 10 years, including primary trisomics for five chromosomes, tetrasomics, double trisomics, triploids, tetraploids, and higher aneuploids (Sharma et al. 1985a, b; Koul et al. 1992).

In one of the aneuploids, initially a primary trisomic for chromosome 2 (Triplo 2), the extra chromosome underwent rapid changes resulting in the formation of ring chromosomes and double minutes and finally was stabilized as an isochromosome. Here we report on the molecular cytogenetic characterization of this isochromosome, which within a few generations has acquired many of the features of a B chromosome.

Materials and methods

Plant material

The *P. lagopus* seeds were originally obtained from the Royal Botanic Gardens, Kew, United Kingdom. Triplo 2 was crossed with euploid *P. lagopus* plants, and a ring chromosome ($2n=12+r$) was identified in addition to the standard chromosome complement in one progeny plant (Fig. 1e) (Dhar 1991). Mitotically this plant was highly unstable and different cells showed different chromosome constitutions including $2n=12+3r+3dm$ (Fig. 1f). The transmission of the ring chromosome was 7.1% and 10.2% through male and female gametogenesis, respectively. In the progeny of this plant, one 13 chromosome plant was identified. This plant had the standard chromosome complement plus an isochromosome ($2n=12+i$). The peculiar characteristics of the isochromosome prompted us to make detailed studies on the nature of this chromosome.

Cytogenetic procedures

Chromosomal constitutions were determined in acetocarmine-stained root-tip meristems. Meiotic metaphase I pairing was determined in pollen mother cells. C-banding was performed according to the standard protocol of Gill et al. (1991) and Ag-NOR banding was according to Dhar et al. (1990). To determine the male and female transmission of the isochromosome, plants with $2n=12+i$ were crossed as males and females with euploid plants

a

**GGTTGCGATCATAACAGCACTAATGCACCGGATCCCATCAGAA
CTCCGCAGTTAAGCGCGCTTGGGCTAGAGCAGTACTAGGATGG
GTGACCCCTCGGAAGTCCCTCGTGTGCAACCCCTTTTTGTCATGT
TCTTGTTCGCGSTTTCCCTTTCTTCGGATCTTTCTTGTCTC
GTTTCTTCCTCCAACACGATACATATTACCTATTCTGGCTGAC
CTCCGTGATATACACAATGTTTTTCGATATCGGGCGTCAACCAC
GTGGAGACGACCGTGTTCGGGGGCGAATTTGACCCCTCGACCTA
AAGGATTTGACCGAAAACGGTTCATCGCTCATGGCGGAGCAATG
ACTTGTGCTCAAAAACCGCTTTCCGATGGAAAACCTATATCGT
TTGAACAAAATCGCACGTTCGGGGGCGTTTTAACATAGGAAGC
CGTGATGAGGCAGCGGGCGGTAAAGGGATAAAATGATAGATTA
CGCATAGGCTATT**

b

**TGGTGCATCATAACAGCACTAATGCACCGGATCCCATCAGAA
CTCCGCAGTTAAGCGCGCTTGGGCTAGAGCAGTACTAAGATGG
GTGACCCCTCGGAAGTCCCTCGTGTGCAACCCCTTTTTTGTGC
TCTTCCGCTCTTATTTTTTAATTTTGGCTTTCTTCTTGTAGT
TTTTCTTCAATCATCTTGGGTAACACATTCGATCGCCATGTG
TTCTATTAAGTCTGTGATTTACGACAAGAATTTGTGTGAATGTG
ACACGTGTTCGGGGCGTATTGGTTCGATTTGCAGCTGACGATTG
AAATTTGGAATCGAAAAGCGCTTCGTTCCGTAATAGATATAAG
AATAGTGCAGCAAGACTAA**

Fig. 2 a DNA sequence of the clone pPov1 containing the 5S rDNA repeat unit of *Plantago ovata* inserted into plasmid pT-Adv; the insert comprises 486 bp of which the first 119 bp constitute the 5S gene (**bold**) and the remaining 367 bp represent the non-transcribed spacer; **b** DNA sequence of the 5S rDNA repeat unit of *Plantago lagopus*; the first 119 bp constitutes the 5S gene (**bold**) and the remaining 234 bp represents the non-transcribed spacer; putative autonomously replicating sequences are *underlined*

or were selfed and the chromosome constitutions of the progeny were determined in root-tip meristems.

Fluorescence in situ hybridization (FISH) was according to the protocol of Kynast et al. (2000) using the clones pTa71 (Gerlach and Bedbrook 1979), pAtT4 (Richards and Ausubel 1988), and pPov1 (Genbank Accession number AF464934), and total genomic *P. lagopus* DNA as probes. Clone pTa71 contains a 9.05 kb *EcoRI* fragment of rDNA isolated from bread wheat and recloned in the *EcoRI* site of the vector pUC19. The 9.05 kb fragment is part of the rDNA repeat unit consisting of one copy of 18S rDNA, 5.8S rDNA, 25S rDNA and an intergenomic spacer derived from *Triticum aestivum* L. cv. Chinese Spring. Clone pAtT4 contains a 0.4 kb fragment of the telomeric repeat unit isolated from *Arabidopsis thaliana* that was inserted in the *HindIII/BamHI* restriction site of the vector pSDS13. pPov1 was cloned from *P. ovata* into the plasmid pT-Adv (Clontech, Palo Alto, Calif., USA) and is 486 bp in length; the first 119 bp constitute the 5S gene, and the remaining 367 bp represent the spacer region (Fig. 2). The spacer region has two *MspI* restriction sites and, therefore, digestion with *MspI* generates three fragments of 349, 110, and 28 bp.

Table 1 Male and female transmission determined in crosses with euploid plants of the trisome in Triplo 2 and in $2n+12+i$ plants. {Expected values are given in parenthesis and were calculated based on a 25% transmission frequency of the extra chromosome. Male and female transmission rates were calculated as follows: the

Chromosome constitution	Female transmission		Male transmission		χ^2	P_{DF1}
	$2n=12$	$2n=13$	$2n=12$	$2n=13$		
Triplo 2	12	8 (5)	22	3 (6.25)	1.8	1.7
$2n=13$						7.1
$2n=12+i$	36	26 (15.5)	30	21 (12.75)	5.3	<0.01 <0.025

Clones pTa71 and pAtT4 were labeled directly with tetramethyl-rhodamine-6-dUTP (Roche Diagnostics, Indianapolis, Ind., USA) according to the manufacturer's instructions. Clone pPov1 was labeled with digoxigenin-11-dUTP, and signals were detected using fluorescein isothiocyanate (FITC)-conjugated sheep-anti-digoxigenin antibodies (Roche Diagnostics, Indianapolis, Ind., USA). For reverse genomic in situ hybridization (rGISH), total genomic *P. lagopus* DNA was labeled directly with fluorescein (Amersham Biosciences, Piscataway, N.J., USA) using an excess of clone pPov1 as a blocker. The post-hybridization stringency wash was at 42°C in 2×SSC and 50% formamide. (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate.) The FISH and rGISH signals were visualized with a Zeiss Axioplan microscope equipped with epifluorescence. Images were captured with a SPOT CCD camera using the appropriate SPOT 2.1 software (Diagnostic Instruments) and processed with Photoshop 4.0 software (Adobe Systems, San Jose, Calif., USA). Images were printed on a Kodak ds 8650 PS color printer. Photomicrographs of conventionally stained and C-banded cells were taken with a Zeiss photomicroscope III using Kodak Imagemink HQ microfilm 1461.

Southern blot analysis

For Southern blot analysis, genomic DNA of euploid *P. lagopus* and the $2n=12+i$ chromosome plants was digested with the restriction enzymes *BamHI*, *ScalI*, and *MspI* and probed with clone pPov1 according to Sambrook et al. (1989) and Qi et al. (1997).

RNA blot analysis

For RNA isolation, we used RNazol (Invitrogen, Carlsbad, Calif., USA) following the manufacturer's instructions. RNA blotting was done by the standard protocols outlined in Sambrook et al. (1989).

Results

The morphology of euploid and $2n=12+1i$ or $2i$ chromosome plants is very similar, indicating that the presence or absence of the isochromosomes does not affect plant growth habit or vigor (Fig. 1a).

C-banding analysis revealed mostly euchromatic A chromosomes with centromeric bands. However, the isochromosome was darkly stained throughout its entire length, indicating that it is completely heterochromatic (Fig. 1c, d).

Meiotic metaphase I pairing analysis of $2n=12+i$ chromosome plants showed that the extra chromosome does not form chiasmate associations with any chromosome of the standard complement. Occasionally, the two

progeny of the cross $2n=12+i$ (female) × $2n=12$ (male) consisted of 36 $2n=12$ and 26 $2n=12+i$ plants resulting in a 42% [12/(36+26)/100] transmission frequency of the isochromosome through the female. The expected frequency of 15.5 was calculated on the basis that the isochromosome is excluded in 75% of the gametes.}

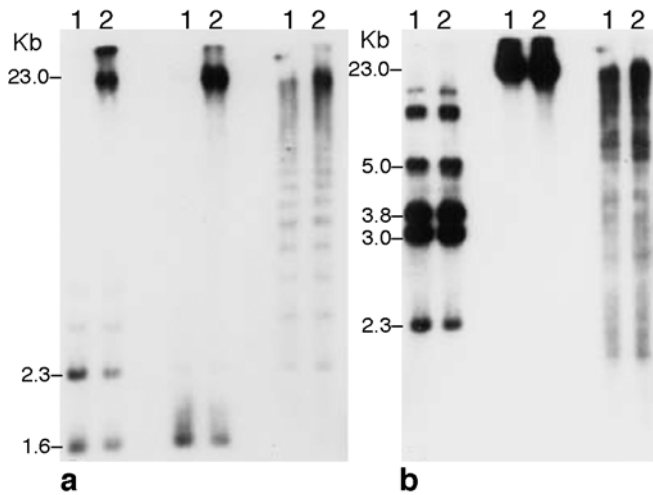


Fig. 3 Southern blot analysis using clones pPov1 (a) and pTa71 (b) to probe *Bam*HI (left), *Sca*I (middle), and *Msp*I (right) digested genomic DNA of *Plantago lagopus* plants with $2n=12$ (1) and $2n=12+i$ chromosomes (2). Note the presence of a distinct block of DNA on top of the lane containing digested DNA from $2n=12+i$ chromosome plants in a, whereas no differences in hybridization patterns were observed between $2n=12$ and $2n=12+i$ chromosome plants using the clone pTa71 (b)

arms of the extra chromosome were paired in the form of a ring univalent, confirming that the extra chromosome was an isochromosome. At anaphase I, the isochromosome segregates to one spindle pole and at anaphase II it divides into two chromatids that segregate to opposite spindle poles.

The transmission of the isochromosome in reciprocal crosses with euploid plants was determined to be 41% and 42% through male and female gametes, respectively (Table 1).

Southern blot analysis of euploid *P. lagopus* and $2n=12+i$ chromosome plants using 5S rDNA clone pPov1 revealed a distinct band at the top of the lane at about 23 kb that is present only in $2n=12+i$ chromosome plants (Fig. 3a). To confirm that this additional band does not represent undigested DNA, the same blot was probed with rDNA clone pTa71. No differences in hybridization patterns were observed between euploid and $2n=12+i$ chromosome plants (Fig. 3b), indicating that the additional 5S rDNA band was derived from the isochromosome.

Fluorescence in situ hybridization analysis using 5S rDNA clone pPov1 as a probe detected one FISH site close to the centromere on one chromosome pair of the standard chromosome complement of *P. lagopus*. The isochromosome was painted over its entire length (Fig. 4a). Fluorescence in situ hybridization results

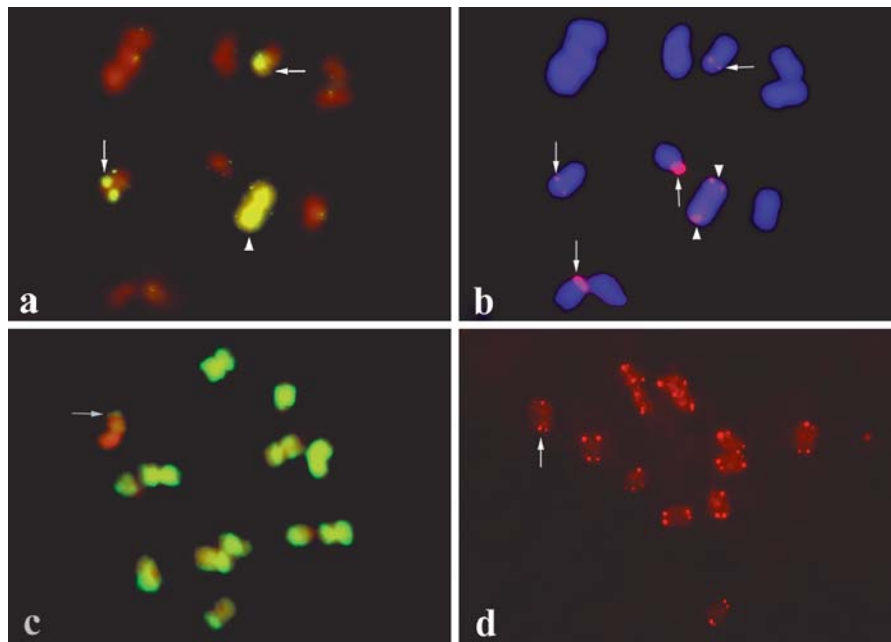


Fig. 4 Fluorescence in situ hybridization (FISH) patterns of $2n=12+i$ mitotic metaphase chromosomes of *Plantago lagopus* using clone pPov1 detected by yellow fluorescein isothiocyanate (FITC) fluorescence (a), pTa71 detected by red rhodamine fluorescence (b), pAtT4 detected by red rhodamine fluorescence (d), and total genomic *P. lagopus* DNA detected by yellow FITC fluorescence in combination with an excess of unlabeled pPov1 DNA (c) as probes: a one pPov1 FISH site is present in one chromosome pair of the standard chromosome complement (arrows), whereas this clone painted the isochromosome over its

entire length (arrowhead); b one major and one minor pTa71 FISH site is present on the standard chromosome complement (arrows) and additional pTa71 FISH sites are present at the terminal regions of both arms of the isochromosome (arrowheads); c note that the isochromosome (arrow) is the only chromosome that is not painted when total genomic *P. lagopus* DNA was used as a probe in combination with pPov1 as blocker; d all chromosomes of the standard complement and also the isochromosome (arrow) have telomeric pAtT4 FISH sites at their termini

Table 2 Nucleolar activity of $2n=12$ and $2n=12+i$, chromosome plants of *Plantago lagopus* (percentages are given in parenthesis)

Chromosome Constitution	Number of nucleoli per cell					
	1	2	3	4	5	6
$2n=12$	21 (36.2)	20 (34.4)	11 (18.9)	6 (10.3)		
$2n=12+i$	14 (19.2)	22 (30.1)	15 (20.5)	14 (19.2)	6 (8.2)	2 (2.7)

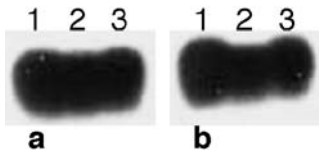


Fig. 5 RNA blot analysis of euploid (lane 1), $2n=12+i$ (lane 2), and $2n=12+2i$ (lane 3) chromosome plants of *Plantago lagopus* sequentially probed with clone pPov1 (a) and an actin clone (b) used as a control. Note that plants with one or two doses of the isochromosome have similar levels of 5S rRNA expression when compared with euploid plants, indicating that the 5S rDNA loci on the isochromosome are not transcribed

confirmed Southern analysis, indicating that the bulk of the isochromosome consists of 5S DNA.

Sequential probing of the same mitotic metaphase with 18S, 5.8S, 25S rDNA clone pTa71 detected two nucleolar organizer region (NOR) loci (one major and one minor) on two chromosome pairs of the standard chromosome complement. The major NOR site is located at the terminal region of the short arm of one chromosome pair. The minor NOR site mapped adjacent to the 5S rDNA site close to the centromere (Fig. 4b) on another pair of chromosomes. Additional NOR loci are present at the termini of each arm of the isochromosome (Fig. 4b).

To verify further the structural organization of the isochromosome, rGISH was performed using total genomic DNA of *P. lagopus* as a probe and either the complete clone pPov1 or only the coding part of the repeat unit as a blocker. In both experiments, all chromosomes of the standard complement were completely labeled, whereas the entire isochromosome had reduced labeling, suggesting that the isochromosome mostly consists of tandemly repeated 5S rDNA units (including the coding and non-transcribed spacer) (Fig. 4c).

Two pairs of Ag-NOR bands were detected in euploid *P. lagopus* plants. Two additional Ag-NOR bands were observed at the ends of both arms of the isochromosome in $2n=12+i$ chromosome plants (data not shown). Similarly, the maximum number of nucleoli in euploid plants was four, whereas it was six in $2n=12+i$ chromosome plants (Table 2), indicating that the additional NORs of the isochromosome are transcriptionally active in organizing nucleoli.

To determine the expression of 5S rDNA loci of the isochromosome, RNA blots of euploid, $2n=12+i$, and $2n=12+2i$ chromosome plants were probed sequentially with clone pPov1 (Fig. 5a) and an actin clone (Fig. 5b) used as a control. The results indicate that the 5S rDNA loci of the isochromosome were not active.

Fluorescence in situ hybridization analysis using telomere-specific clone pAtT4 as a probe revealed the expected FISH sites at the telomeres of all chromosomes of the standard complement. Fluorescence in situ hybridization signals were also observed on the telomeres of both arms of the isochromosome (Fig. 4d), providing convincing evidence for a fully functional and stable chromosome.

Discussion

B chromosomes have been defined as “dispensable supernumeraries which do not recombine with any members of the basic A chromosome set and have irregular and non-Mendelian modes of inheritance” (Jones 1995). B chromosomes occur in all eukaryotic organisms and in 10%–15% of flowering plants and appear to be restricted to outbreeders. They are usually smaller but some may be as large as or larger than A chromosomes. In morphology, Bs may exist as isochromosomes, acrocentrics or telocentrics. They may be totally or partially heterochromatic, usually genetically inert, but may have NOR, 5S rDNA and other genes that affect recombination and ensure their preferential transmission through the gametes (Beukeboom 1994; Jones 1995). The newly arisen isochromosome described here for *P. lagopus* has many properties of B chromosomes. As such it should be considered a bona fide B chromosome with a fully documented mode of origin.

Chromosome structure and behavior

The data show that the isochromosome is cytogenetically unique. It is the largest chromosome, more than twice the size of the A chromosomes of *P. lagopus*. C-banding analysis showed that the isochromosome is completely heterochromatic. It has NOR loci at the ends and the bulk of the body mass is derived from 5S rDNA sequences. The ends of the chromosome have been stabilized by telomeric sequences. It has a fully functional centromere as judged from its regular behavior during mitosis and meiosis. During meiosis, it does not pair with any chromosome of the standard complement. Instead, the two arms of this chromosome are paired internally, resulting in a ring univalent. It undergoes normal disjunction at meiosis I and II. The structure and behavior are typical of many naturally occurring B chromosomes. For example, the much studied B chromosomes of *Crepis capillaris* exist as isochromosomes and also have NOR

and 5S rDNA loci (Jones 1995). However, the isochromosome described here is unique in having its entire body mass derived from 5S DNA sequences. No plant B chromosome with such a structure has been described, partly because not all B chromosomes have been analyzed for the presence of 5S rDNA sequences. However, in *Rattus* it has been reported that ribosomal cistrons are dispersed throughout the B chromosomes (Stitou et al. 2000).

Genetic activity

Most B chromosomes in nature are described as genetically inert although they may carry one or more traits unique to their survival (Jones 1995). The isochromosome described here is also genetically inert as indicated by its completely heterochromatic structure, and lack of visible effects on plant morphology and vigor. Because of genetic unbalance, pollen (male gametophyte) is very sensitive to aneuploidy. As a rule, unbalanced male gametes ($1n+1$) cannot compete against euploid ones ($1n$) and extra chromosomes are transmitted at a reduced rate through males (Khush 1973). In Triplo 2, from which the isochromosome originated by fragmentation, $1n+1$ male gametes function at a frequency of 12% (Bahn 1990) as compared with the 41% for the isochromosome (Table 1). This is the most compelling evidence that the isochromosome is genetically inert. However, the NOR loci on the isochromosome do organize nucleoli as has also been documented for B chromosomes in *C. capillaris* and other organisms (Jones 1995).

B chromosome drive

Many drive and accumulation mechanisms have been documented by which B chromosomes enhance their transmission and accumulate in nature (Jones 1991, 1995). These include premeiotic, meiotic or postmeiotic drive, generally controlled by discrete genetic factors located on the B chromosomes. B chromosome drive or accumulation mechanisms may operate in the male or female side or rarely through both sexes or even can be absent in some cases. Thus, of the 70 species of plants analyzed, only 60% have shown evidence of meiotic drive (Jones 1995). The B chromosome accumulation mechanism is usually associated with B chromosome nondisjunction during pre- or postmeiotic mitosis and B chromosome carrier germ cells usually participate in gametogenesis and fertilization. Meiotic drive involves directed movement of B chromosomes to poles to minimize their loss during meiosis or to egg cells that will participate in zygotic fertilization. We have not analyzed details of the drive behavior of the isochromosome reported here but the data do suggest preferential transmission. B chromosomes do not pair with A chromosomes and form univalents that can be described as laggards. There is an extensive body of literature

documenting that a laggard (unpaired or univalent) chromosome is transmitted to 25% of the gametes by chance and 75% of the time it is lost as micronuclei (Sears 1944). The observed rate of 40%–41% in both sexes is significantly higher than 25% (Table 1). Thus, even though only 25% of gametes are expected to have the B chromosome, 40% gametes with Bs were actually involved in fertilization, indicating the existence of a drive mechanism. In maize controlled crosses, male ($0B \times 1B$) and female ($1B \times 0B$) transmission of Bs was 38% and 30%, respectively (data of Randolph 1941 cited in Jones 1995). In maize, the drive mechanism only operates on the male side with a well-known mechanism of accumulation caused by B chromosome nondisjunction at the second pollen mitosis.

Mode of origin

Although a lot of work has been conducted on the structure, occurrence, sequence analysis, meiotic behavior and transmission of B chromosomes, their origin is still an enigma (Jones 1995; Langdon et al. 2000). It is widely accepted that B chromosomes originate from standard A chromosomes. In addition to this intraspecific mode of origin, there also is evidence suggesting that B chromosomes are alien chromosomes introgressed from related species by interspecific hybridization (Camancho et al. 2000). B chromosomes likely originate by several mechanisms. We were able to observe one such rare event and document one of the mechanisms by which a B chromosome may arise in nature.

From our data, we can deduce several distinct processes that may participate in the origin of a B chromosome. The first process is a mutation causing aneuploidy. Aneuploids arise spontaneously from chromosome nondisjunction and other causes (Khush 1973). Aneuploidy may play a role in the origin of B chromosomes by providing the raw material, i.e., the centromere and autonomously replicating DNA sequences (ARs), and also may trigger aneuploidy-associated genetic lesions such as chromosome fragmentation. However, any mutation in euploid plants that causes chromosome fragmentation can serve the same purpose because centromeres have been shown to be divisible into functional subunits (Zhang et al. 2001). Amplification of DNA sequences then occurs to provide body mass because a certain minimum size is required for a functional chromosome (Schubert and Oud 1997). As discussed below, several mechanisms are known that can lead to rapid amplification of DNA sequences. The acquisition of telomeric DNA sequences to stabilize the chromosome end is the final process (Werner et al. 1992; Friebe et al. 2001).

The results of this study show that all of these processes participated in the origin of the isochromosome reported here. The isochromosome is composed almost entirely of 5S rDNA sequences, and the two termini of this chromosome have 18S, 5.8S, 25S rDNA sequences

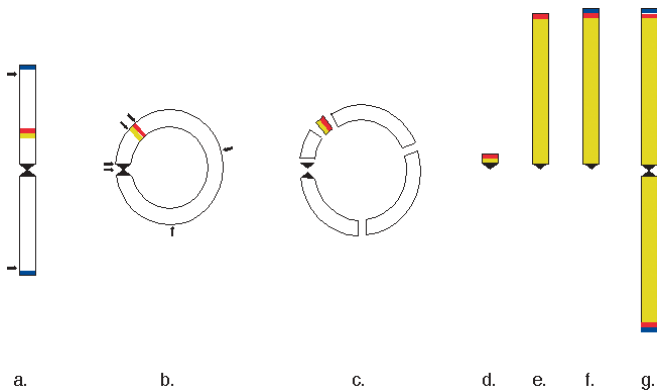


Fig. 6 Possible mode of origin of the isochromosome: **a** in Triplo 2, chromosome 2 with a functional centromere (black), telomere (blue), and adjacent 5S rDNA (yellow) and 18S, 5.8S, 25S rDNA (red) sequences was present in triplicate and vulnerable to chromosome breakage; **b** breaks in distal regions of both arms followed by the fusion of the broken ends produced a ring chromosome; **c** fragmentation of the ring chromosome and **d** fusion of a fragment containing the 5S and 18S, 5.8S, 25S rDNA sequences with a centromeric fragment produced a nascent minichromosome; **e** amplification of 5S rDNA sequences occurred; **f** addition of telomeric repeats stabilized the end of the telosome; **g** misdivision of the centromere resulted in the formation of the observed isochromosome

followed by telomeric repeats. Although we do not know how the isochromosome originated, the following hypothesis is consistent with our observations. The origin of the isochromosome can be traced to chromosome 2, which has tandem arrays of both 5S and 18S, 5.8S, 25S rDNA sequences (Fig. 6a). The $2n=13$ chromosome plant was first isolated in the progeny of Triplo 2, indicating that the first step was the aneuploid mutation that triggered the chain of events leading to the creation of a stable isochromosome. Because chromosome 2 was present in triplicate in the mother plant, it was probably more vulnerable to chromosome breakage. The second step was the formation of a ring chromosome that resulted from two breaks in both arms of chromosome 2 (Fig. 6b). The third step involved chromosome fragmentation (Fig. 6c). Perhaps several double-strand breaks occurred in chromosome 2, which resulted in a derivative nascent minichromosome containing both the 5S and 18S, 5.8S, 25S rDNA arrays fused to centromeric sequences (Fig. 6d). The observation of ring chromosomes and double minutes during the development of this line supports this hypothesis. The stage was set for the fourth process: DNA sequence amplification (Fig. 6e).

In plants, gene amplification has been reported to occur during evolution (Bedbrook et al. 1980), in *in vitro* plant culture (Phillips et al. 1994) or in response to metabolic stress (Donn et al. 1984). Furthermore, the response of genomes to stress has been amply demonstrated in maize (McClintock 1984). Rapid chromosome remodeling was also observed in interspecific hybrids of marsupials (Waugh O'Neill et al. 1998). The centromeres of one parental genome in these hybrids became elongated as the result of genome-wide undermethylation that

led to the amplification of retroelements and their insertion into the centromeric heterochromatin followed by additional chromosomal rearrangements. Similarly, the challenges posed by trisomy in *P. lagopus* may have triggered the excision and amplification of 5S rDNA sequences.

DNA sequences can be amplified by different mechanisms in different organisms. In addition to classical examples such as amplification of rRNA genes in amphibian oocytes, macronuclei of *Tetrahymena*, and chorion genes in *Drosophila*, cells with amplified oncogenes and drug-resistance genes have been observed both *in vivo* in tumors and in cell lines (Schimke 1988). Although several models for gene amplification have been proposed (Windle and Wahl 1992), none of them seem universally acceptable. Our data also are not consistent with any single model of gene amplification. Most likely, unscheduled DNA replication, termed Onion-Scale replication (Schimke et al. 1986) gave rise to amplified 5S rDNA arrays.

The fifth process was the addition of telomeric repeats to the end that stabilized this chromosome (Fig. 6f). This probably happened in the germ cells where telomerase activity is known to be high (Eisenhauer et al. 1997). It is tempting to speculate that enhanced nucleolar activity may have played a role in the preferential transmission of the newly arisen isochromosome as NOR loci have been documented in many of the naturally occurring B chromosomes (Jones 1995). Finally, misdivision of the centromere (Rhoades 1938; Sears 1952a, b) occurred that led to the formation of the isochromosome (Fig. 6g).

For yet another reason, the involvement of rRNA genes in the amplification process does not appear to be accidental. Recently, Borisjuk et al. (2000) cloned an amplification-promoting sequence (aps) from the intergenic spacer of rDNA in *Nicotiana*. This sequence stimulates the amplification of adjacent genes. This is particularly true for an 11 bp core consensus sequence (A/T)TTTAT(A/G)TTT(A/T) of the *Saccharomyces cerevisiae* ARS that was found within the origins of DNA replication in several eukaryotes including plants (Hernandez et al. 1993). Using the CLUSTAL W (<http://dot.imgen.bcm.tmc.edu>) computer program, we aligned the aps sequence with the internal transcribed spacer (ITS) and non-transcribed spacer (NTS) sequences of the 5S rRNA gene cloned from *P. lagopus* (Fig. 2b) (Accession number AF464934). No significant matches were found between the aps and ITS sequences although the aps and NTS sequences showed about 50% similarity. Interestingly, three sequences, TTTTAAATTTT, ATGT-GTTCTATTA, and ATTTGGAAT, showed considerable similarity to ARS sequences. The first sequence shows 10/11 matches with the ARS sequences, and the other two sequences show 12/13 and 9/10 matches. The mouse muNTS1 and several other mammalian aps elements also contain ARS sequences, which suggests that ARS-like elements are required for gene amplification in both mammals and plants. The presence of an ARS-like sequence in the NTS region of the 5S rRNA gene

suggests its involvement in the amplification of 5S rDNA arrays that led to the origin of the isochromosome characterized in this study.

Fate of the apparent B chromosome

The isochromosome reported here already has acquired many characteristics that qualify it as a B chromosome but it is still in the very early stages of B chromosome evolution. It will be expected to evolve a more potent drive and accumulate DNA sequences by horizontal transfer or otherwise lose DNA sequence similarity to A genome chromosomes. Green (1990) has suggested that B chromosomes undergo degeneration by Muller's ratchet mechanism. These processes are related to the peculiar behavior of B chromosomes, similar to the Y chromosome of mammals (Graves 1995), because of the absence of pairing and recombination.

Acknowledgements The senior author thanks the Department of Science and Technology, Government of India for a BOYSCAST fellowship and the Wheat Genetics Resource Center at Kansas State University for providing laboratory facilities. This research was partly supported by a special USDA grant to the Wheat Genetics Resource Center. This paper is contribution number 02184-J from the Kansas Agricultural Experiment Station, Kansas State University, Manhattan, KS 66506-5502, USA.

References

- Bedbrook JR, O'Dell M, Flavell RB (1980) Amplification of rearranged repeated DNA sequences in cereal plants. *Nature* 288:133–137
- Beukeboom LW (1994) Bewildering B's: an impression of the 1st B-chromosome conference. *Heredity* 73:328–336
- Bhan A (1990) Cytogenetic analysis of aneuploids of *Plantago lagopus*. PhD Thesis, University of Jammu, Jammu, India
- Borisjuk N, Borisjuk L, Komarnytsky S, Timeva S, Hemleben V, Gleba Y, Raskin I (2000) Tobacco ribosomal DNA spacer element stimulates amplification and expression of heterologous genes. *Nature Biotechnol* 18:1303–1306
- Camancho JPM, Sharbel TF, Beukeboom LW (2000) B-chromosome evolution. *Philos Trans R Soc London B Biol Sci* 355:163–178
- Dhar MK (1991) Cytogenetic studies on some telo aneuploids of *Plantago lagopus* and their progeny. PhD Thesis, University of Jammu, Jammu, India
- Dhar MK, Koul AK, Langer (1990) NOR in *Vicia faba*. *Chromosome Inf Serv* 49:8–20
- Donn G, Tischer E, Smith JA, Goodman HM (1984) Herbicide resistant alfalfa cells: an example of gene amplification in plants. *J Mol Appl Genet* 2:621–635
- Eisenhauer KM, Gerstein RM, Chiu C-P, Conti M, Hsueh AJW (1997) Telomerase activity in female and male rat germ cells undergoing meiosis and in early embryos. *Biol Reprod* 56:1120–1125
- Friebe B, Kynast RG, Zhang P, Dhar M, Gill BS (2001) Chromosome healing by addition of telomeric repeats in wheat occurs during the first divisions of the sporophyte and is a gradual process. *Chromosome Res* 9:137–146
- Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. *Nucleic Acids Res* 7:1869–1885
- Gill BS, Friebe B, Endo TR (1991) Standard karyotype and nomenclature system for description of chromosome bands and structural aberrations in wheat (*Triticum aestivum*). *Genome* 34:830–839
- Graves JAM (1995) The origin and the function of the mammalian Y chromosome and Y-chromosome genes. An evolving understanding. *BioEssays* 17:311–320
- Green DM (1990) Muller's ratchet and the evolution of sex chromosomes. *Genome* 33:818–824
- Hernandez P, Martin-Parras L, Martinez-Robles ML, Schwartzman JB (1993) Conserved features in the mode of replication of eukaryotic ribosomal RNA genes. *EMBO J* 12:1475–1485
- Jones RN (1991) B-chromosome drive. *Am Nat* 137:430–442
- Jones RN (1995) B chromosomes in plants. (*Tansley Rev No* 85) *New Phytol* 131:411–434
- Jones RN, Rees H (1982) B chromosomes. Academic Press, London, pp 266
- Khush GS (1973) Cytogenetics of aneuploids. Academic Press, New York
- Koul AK, Dhar MK, Bhan A, Langer A (1992) Tolerance of *Plantago lagopus* to chromosome engineering. *Perspect Cytol Genet* 7:591–605
- Kynast RG, Friebe B, Gill BS (2000) Fate of multicentric and ring chromosomes induced by a new gametocidal factor located on chromosome 4M^s of *Aegilops geniculata*. *Chromosome Res* 8:133–139
- Langdon T, Seago C, Jones RN, Ougham H, Thomas H, Forster JW, Jenkins G (2000) De novo evolution of satellite DNA on the rye B chromosome. *Genetics* 154:869–884
- McClintock B (1984) The significance of responses of the genome to challenge. *Science* 226:792–801
- Phillips RL, Kaeppler SM, Olhoft P (1994) Genetic instability of plant tissue culture: breakdown of normal controls. *Proc Natl Acad Sci U S A* 91:5222–5226
- Qi LL, Wang SL, Chen PD, Liu DJ, Friebe B, Gill BS (1997) Molecular cytogenetic analysis of *Leymus racemosus* chromosomes added to wheat. *Theor Appl Genet* 95:1084–1091
- Rhoades MM (1938) On the origin of a secondary trisome through the doubling of a half-chromosome fragment. *Genetics* 23:163–164
- Richards EJ, Ausubel FM (1988) Isolation of a higher eukaryotic telomere from *Arabidopsis thaliana*. *Cell* 53:127–136
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- Schimke RT (1988) Gene amplification in cultured cells. *J Biol Chem* 263:5989–5992
- Schimke RT, Sherwood SW, Hill AB, Johnston RN (1986) Overreplication and recombination of DNA in higher eukaryotes: potential consequences and biological implications. *Proc Natl Acad Sci U S A* 83:2157–2161
- Schubert I, Oud JL (1997) There is an upper limit of chromosome size for normal development of an organism. *Cell* 88:515–520
- Sears ER (1944) Cytogenetic studies with polyploid wheat species. II. Additional chromosomal aberrations in *Triticum vulgare*. *Genetics* 29:232–246
- Sears ER (1952a) Misdivision of univalents in common wheat. *Chromosoma* 4:535–550
- Sears ER (1952b) The behavior of isochromosomes and telocentrics in wheat. *Chromosoma* 4:551–562
- Sharma PK, Koul AK (1984) Genetic diversity among *Plantagos* III. Primary trisomy in *Plantago lagopus* L. *Genetica* 64:135–138
- Sharma PK, Langer A, Koul AK (1985a) Genetic diversity among *Plantagos* V. Transmission of the additional chromosome in a triplo 4 individual of *Plantago lagopus* L. *Genetica* 67:131–135
- Sharma PK, Langer A, Koul AK (1985b) Genetic diversity among *Plantagos* VII. Nature of aneuploids in the progeny of aneutriploid × diploid plants of *Plantago lagopus* L. *Genetica* 67:137–144
- Stitou S, Jimenez R, Diaz de la Guardia R, Burgos M (2000) Inactive ribosomal cistrons are spread throughout the B

- chromosomes of *Rattus rattus* (Rodentia, Muridae). Implications for their origin and evolution. *Chromosome Res* 8:305–311
- Waugh O'Neill RJ, O'Neill MJ, Graves JA (1998) Undermethylation associated with retroelement activation and chromosome remodeling in an interspecific mammalian hybrid. *Nature* 393:68–72
- Werner JE, Kota RS, Gill BS (1992) Distribution of telomeric repeats and their role in the healing of broken chromosome ends in wheat. *Genome* 35:844–848
- Windle BE, Wahl GM (1992) Molecular dissection of mammalian gene amplification: new mechanistic insights revealed by analyses of very early events. *Mutat Res* 276:199–224
- Zhang P, Friebe B, Lukazewski AJ, Gill BS (2001) The centromere structure in Robertsonian wheat-rye translocation chromosomes indicate that centric breakage-fusion can occur at different places within the primary constriction. *Chromosoma* 110:335–344