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DNA content of the B chromosomes in grasshopper *Podisma kanoi* Storozh. (Orthoptera, Acrididae)

Alexander G. Bugrov[1,2]*, Tatiana V. Karamysheva[3], Eugeny A. Perepelov[1,3], Eugeny A. Elisaphenko[3], Denis N. Rubtsov[1], Elżbieta Warchałowska-Śliwa[4], Haruki Tatsuta[5] & Nikolay B. Rubtsov[1,3]
[1]*Novosibirsk State University, 630090 Novosibirsk, Russia; Tel: +7(383)3397564; Fax: +7(383)3397564; E-mail: bugrov@fen.nsu.ru;* [2]*Institute for Systematics and Ecology of Animals, Siberian Branch of Russian Academy of Sciences, 630091 Novosibirsk, Russia;* [3]*Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, 630090 Novosibirsk, Russia;* [4]*Department of Experimental Zoology, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016 Kraków, Poland;* [5]*National Institute for Environmental Studies, 16-2, Onogawa, Tsukuba, Ibaraki 305-8506, Japan*
** Correspondence*

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Abstract

A DNA library derived from the B chromosome of *Podisma kanoi* was obtained by chromosome microdissection. A total of 153 DNA clones were isolated from the microdissected DNA library. Twenty of them were sequenced. A comparison of B chromosome DNA sequences with sequences of other species from the DDBJ/GenBank/EMBL database (http://www.ddbj.nig.ac.jp/) was performed. Different patterns of signals were observed after FISH with labeled cloned DNA fragments. FISH signals with cloned DNA fragments painted either whole Bs or their different regions. Some clones also gave signals in pericentromeric regions of A chromosomes. Other cloned DNA fragments gave only background-like signals on A and B chromosomes. Comparative FISH analysis of B chromosomes in *Podisma kanoi* and *P. sapporensis* with DNA probes derived from the Bs of these species revealed homologous DNA that was confined within pericentromeric and telometric regions of the B chromosome in *P. kanoi*. In contrast to the B chromosomes in *P. sapporensis* containing large regions enriched with rDNA, only a small cluster of rDNA was detected in one of the examined B chromosomes in *P. kanoi*. The data strongly suggest an independent origin of B chromosomes in two closely related *Podisma* species.

Introduction

Extra chromosomes (Bs) to the standard complement (A) have been reported in many species. Extensive studies have been performed on Bs, including analysis of B chromosome distribution across species, the structure and origin of Bs, effects of Bs on the standard complement, transmission of Bs, population dynamics and evolution, and integration of Bs into the A genome (Camacho *et al.* 2000); but little is known about the DNA content of Bs. The B chromosomes in grasshoppers are relatively well studied. High morphological diversity in size and location of C-negative and C-positive regions in Bs, and regions enriched with rDNA and satellite repeats, have been described for *Eyprepocnemis plorans* (Camacho *et al.* 1997a,b, Henriques-Gil *et al.* 1984, Henriques-Gil & Arana 1990, Bakkali *et al.* 1999, Cabrero *et al.* 1999). Despite similar DNA content, the origin of at least

two types of Bs in this species appeared to be independent (Cabrero *et al*. 2003). A partial sequence of the B chromosome in *E. plorans* has been obtained (data presented by Professor J. P. Camacho at the Second B Chromosome Conference, Bubion, Granada, Spain, 26–29 June, 2004).

An extensive study of Bs was performed in another group of grasshopper species belonging to the genus *Podisma*. In *P. sapporensis* Shir., cytological variation of B chromosomes has been found in some populations in Hokkaido, Sakhalin and Kuril islands. The Bs of this species have been divided into seven morphotypes according to their size, morphology and C-banding patterns (Warchałowska-Śliwa *et al*. 2001). A DNA library derived from the B of *P. sapporensis* Shir. (morphotype B1) was generated by chromosome microdissection followed with DOP-PCR and used in a study on B chromosome organization and distribution of homologous DNA in A and B chromosomes of *Podisma* species (Bugrov *et al*. 2003, 2004). The comparative FISH study on the Bs of other morphotypes revealed regions containing DNA showing no homology to the B1 chromosome. In some Bs only small regions have shown signals after FISH with a microdissected DNA probe derived from the B1 chromosome (Bugrov *et al*. 2004). An analysis of repetitive DNA distribution in A and B chromosomes suggested that B chromosome evolution in *P. sapporensis*, at least in some cases, was mainly associated with DNA insertions into an original A chromosome followed by amplification of inserted DNA and deletion of some ancestral chromosome regions (Bugrov *et al*. 2004).

The diversity of molecular organization of the Bs in *P. sapporensis* raised new questions on the mechanisms of B chromosome origin and organization in closely related species belonging to the genus *Podisma*. A comparative analysis of morphology and molecular organization of B chromosomes in the *P. sapporensis* group distributed in Sakhalin, Kunashiri, Hokkaido and Honshu Islands would be of great help to understand the further evolution of B chromosomes and mechanisms that may be involved in these processes. The present study is a detailed analysis of the B chromosome in *P. kanoi* and a comparison of B chromosome organization in two closely related species, *P. kanoi* and *P. sapporensis*. An analysis of repetitive DNA distribution in A chromosomes of these species was also performed.

Material and methods

Samples

During July–August 2004 and 2005 a total of 39 females and 77 males of *P. kanoi* Storozhenko, 1993 were collected from three sites on Honshu Island (Mt Shirane, Manza, and Mt Naeba). Females were placed in separate cages according to locality. After a few days the females laid egg pods in moist, coarse sand. All males and 53 embryos were used for cytogenetic analysis. The specimens of *P. sapporensis sapporensis* captured from populations in Hokkaido Island have been described earlier (Bugrov *et al*. 2003).

Chromosome analysis

Chromosomes for routine cytogenetic analysis and FISH were prepared from embryos and testes of adult males as described by Bugrov *et al*. (2000, 2004). Males were injected with 0.1% colchicine for 1.5–2.0h. The testes were fixed in alcohol:glacial acetic acid (3:1) for 15min, and then kept in 70% ethanol. Air-dried preparations were prepared by squashing the testicles in 45% acetic acid and freezing in dry ice. Each portion of the eggs was stored in a separate Petri dish with moist sand and kept at room temperature. After 15–20 days of incubation the eggs were placed in a solution of 0.05% colchicine in insect saline and the tops of the nonmicropylar end were removed. They were incubated at 30 C^0 for 1.5–2h. The embryos were dissected out of the eggs and placed into a 0.9% sodium citrate solution at room temperature. After 20–30 min they were fixed in 3:1 methanol:acetic acid. Air-dried preparations were prepared on slides by macerating the embryos in a drop of 60% acetic acid.

Meiotic and mitotic chromosomes were stained with Giemsa, DAPI and a standard C-banding technique (Sumner 1972).

DNA probes

Meiotic chromosome microdissection followed with DOP-PCR was used for preparation of DNA probes from the whole *P. kanoi* B chromosome (Bk) and from the central region of the B chromosome arm (Bm). Briefly, dissected regions of eight copies of Bs were transferred into 40nl of buffer solution in a

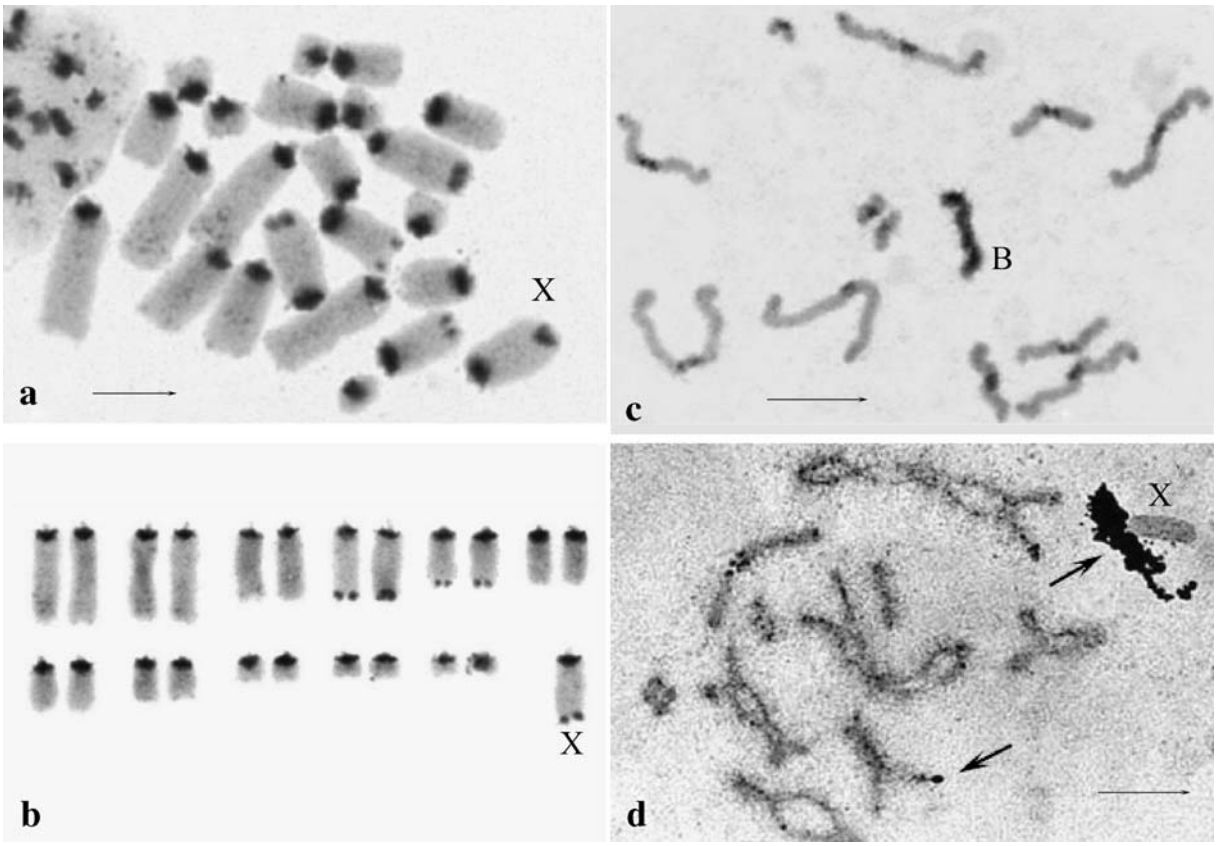

Figure 1. C-banded and NOR karyotypes of *Podisma kanoi*. (**a**) Mitotic metaphase in embryo; (**b**) karyogram of mitotic metaphase; (**c**) metaphase II, indicates B chromosome; (**d**) AgNOR stained metaphase I, arrows indicate NORs. Scale bars = 10μm.

siliconized micropipette tip, treated with proteinase K, and then amplified using DOP-PCR with the MW6 primer (Rubtsov *et al*. 2000, 2002).

The DNA libraries were labeled with biotin-16-dUTP or digoxigenin-11-dUTP in 15 PCR cycles (Rubtsov *et al*. 2000). The DNA probes were used for FISH analysis of meiotic and mitotic chromosomes of *P. sapporensis* and *P. kanoi*. FISH was performed according to a standard protocol (Rubtsov *et al*. 2002) with salmon sperm DNA as the DNA carrier. Biotin- and digoxigenin-labeled DNA probes were visualized with avidin-FITC and mouse antidigoxigenin antibodies conjugated to Cy3, respectively.

Microdissected DNA probes generated from the B chromosome of *P. sapporensis* (B1) and the euchromatic part of the smallest A chromosome (EUR1) (Bugrov *et al*. 2004) were also used for FISH analysis.

Biotin-, digoxigenin-, and dinitrophenyl-labeled, cloned DNA fragments derived from microdissected DNA libraries were obtained by PCR with M13 direct and reverse primers in 25 cycles.

The rDNA-probe was a nick-translation labeled fragment of human 18S rDNA cloned in pHr13 (Malygin *et al*. 1992).

The chromosomes were counterstained with DAPI and analyzed using an AXIOSKOP 2 Plus microscope (Zeiss) equipped with a CCD camera, filter sets and the ISIS3 image-processing package of Metasystems GmbH in the Microscopic Centre of the Institute of Cytology and Genetics SB RAS, Novosibirsk, Russia.

Sequencing of B chromosome DNA

DNA fragments of the B chromosome for dideoxy sequencing were produced with two techniques:

1. DNA fragments from the microdissected *P. kanoi* B chromosome DNA library were ligated to the

XhoI site of the plasmid pBluescript II KS+ and then used for transformation of competent *Escherichia coli* XLI-Blue cells. Colonies of recombinants were selected by their color.
2. Alternatively the pCR4Blunt-TOPO vector with the TOPO Shotgun Subcloning Kits (Invitrogen) were used according to the manufacturer's recommendation. Recombinant colonies were selected on ampicillin plates.

The cloned DNA fragments were sequenced with 'M13 direct' and reverse primers in both cases. These primers were used to sequence cloned fragments on an Applied Biosystems 371A Automated DNA Sequencer utilizing BigDye terminators (ABI PRISM).

The BLAST and FASTA packages were used for computer analysis of obtained sequences. A homology search was done among the sequences of the DDBJ (http://www.ddbj.nig.ac.jp/).

Results

C- and Ag-NOR-banding patterns of the A and B chromosomes

The karyotype of *P. kanoi* showed a standard chromosome complement typical for the vast majority of grasshoppers (White 1973, Hewitt 1979), consisting of 22 acrocentric autosomes plus a single acrocentric X chromosome in males (2n ♂=23, X0) and two Xs in females (2n ♀=24, XX) (Figure 1a,b). Pericentromeric C-blocks were found in all chromosomes of the set. These blocks are large, and approximately uniform in size. All A chromosomes possessed extremely short arms. The X and two pairs of the medium-sized autosomes have C-positive blocks in their telomeric regions (Figure 1a,b). Active NORs were detected with Ag-NOR-banding. A larger NOR was localized in the telomeric region of the X, a smaller one was found in the telomeric region of a medium-sized autosome (Figure 1c).

Five out of 51 samples from the Mt Shirane population were characterized by the presence of B chromosomes. Three samples with Bs were found among 17 samples from Mt Naeba. Bs were not found in the Manza population. All B chromosomes showed a similar morphology, i.e. being acrocentrics of medium size (Figure 1d) with C-positive staining. No C-negative region was revealed in *P. kanoi* Bs, in contrast to the Bs in *P. sapporensis* (Bugrov *et al.* 2003, 2004).

Chromosome polymorphism in the population of *P. s. sapporensis* and *P. s. krylonensis* has been described earlier (Bugrov *et al.* 2001, 2004, Warchałowska-Śliwa *et al.* 2001) in detail.

FISH with microdissected DNA probes on A and B chromosomes in P. kanoi *and in* P. sapporensis

FISH with the B1 probe derived from a *P. sapporensis* B1 chromosome on the Bs in *P. kanoi* gave signals only in pericentromeric and telomeric regions; both of them were very weak. Some of the A chromosomes showed signals in pericentromeric regions (Figure 2a). The FISH signal was also detected in telomeric regions of the X chromosome and in one pair of autosomes. No C-positive region of A and B chromosomes in either species was painted with the EUR1-probe derived from the euchromatic part of the smallest A chromosome of *P. sapporensis* (Figure 2a), while all C-negative regions of A chromosomes showed a strong signal in the studied species. Taken together, this information encouraged us to prepare two microdissected DNA probes from the *P. kanoi* B chromosome. The first probe (Bk DNA probe) was derived from the whole B chromosome found in the male from the Shirane population. The second probe (Bm DNA probe) was prepared from the middle section of the same B. FISH with the Bk DNA probe gave dot-like signals in short arms and probably in pericentric regions of all A chromosomes (Figure 2b). It should be noted that the short arms of chromosomes in *P. kanoi* are very small. They are almost indiscernible in meiotic chromosomes. Based on the results of FISH experiments with meiotic chromosomes, it is impossible to discriminate the pericentric repeats and subtelomeric repeats of the short arms because in both cases the FISH signal covered the pericentric regions and short arms of chromosomes. The bright painting of the B chromosomes masked the signal on A chromosomes. In order to analyze the FISH signal on A chromosomes, FISH was performed on samples without B chromosomes. These FISH experiments revealed that the signal was more intense on three pairs of autosomes. Furthermore, on these chromosomes FISH with the Bk DNA probe produced signals on larger regions, including the short arms and pericentric regions while

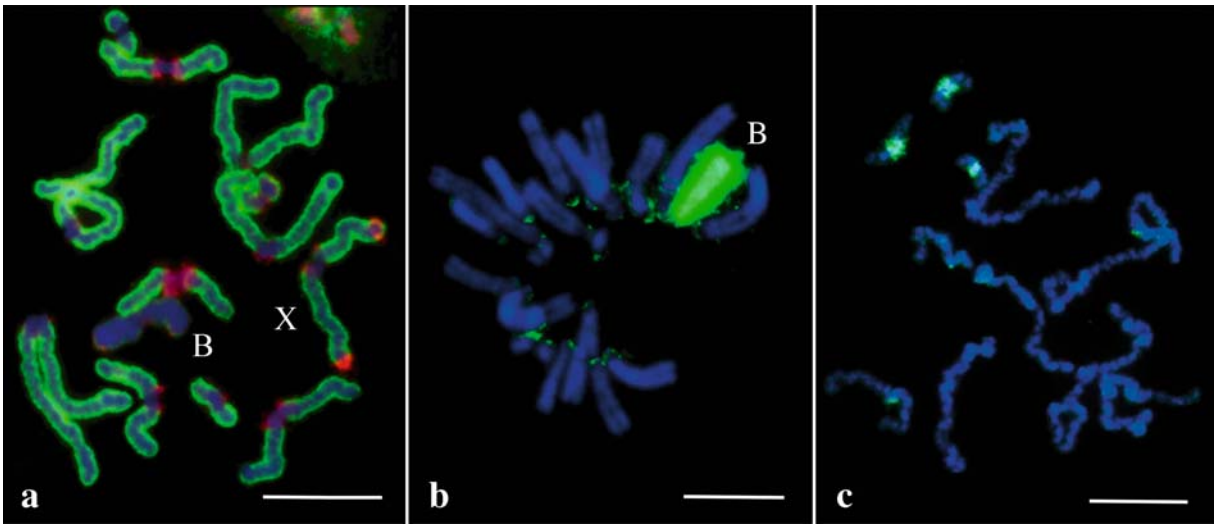

Figure 2. FISH of B1 (red signal) and EUR1 (green signal) probes on *P. kanoi* chromosomes. (**a**) Metaphase II; FISH of Bk- (green signal) probe in *P. kanoi* chromosomes; (**b**) spermatogonial metaphase plate with B chromosome; (**c**) metaphase II without B. X and B indicated. Scale bars = 10 μm.

on other chromosomes only a slight, distant, dot-like signal was registered (Figure 2c). FISH with the B1 probe (DNA probe derived from the *P. sapporensis* B1) gave intensive and widespread signals on pericentric regions of *P. kanoi* A chromosomes. In pericentric regions, the FISH signal of the B1 probe was close to the FISH signal of the Bk and Bm DNA probes. In telomeric regions of long arms of the X and one middle-sized autosome the FISH signals of the B1 probe were co-localized with the FISH signals of the rDNA probe.

FISH with microdissected DNA probes derived from the B chromosome of *P. kanoi* on chromosomes of *P. sapporensis* gave a background-like signal along all chromosomes but no region-specific signal was revealed.

FISH with the rDNA-probe on A and B chromosomes in P. sapporensis *and* P. kanoi

FISH with rDNA on chromosomes of *P. kanoi* gave intense signals in telomeric regions of long arms of the X and one middle-sized autosome (Figure 3a). No signal was detected on *P. kanoi* B chromosomes in any sample except for one collected from the Mt Naeba population (Figure 3b). The intensity of the

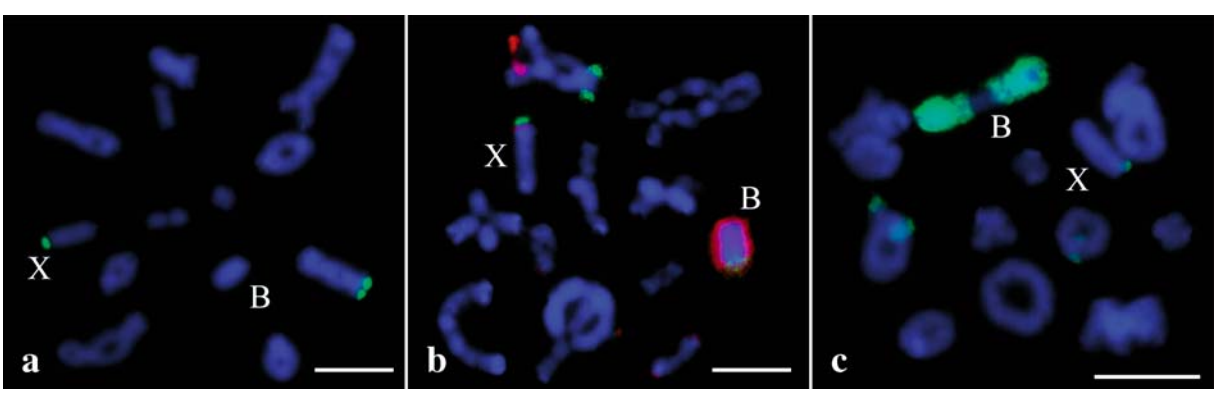

Figure 3. FISH of rDNA probe on *P. kanoi* meiotic chromosomes in metaphase I; X and B indicated. (**a**) Plate with a B chromosome without a NOR; (**b**) FISH of Bk (red signal) and rDNA (green signal) probes on *P. kanoi* meiotic chromosomes; (**c**) FISH of rDNA probe (green signal) in *P. sapporensis* meiotic chromosomes. Scale bars = 10μm.

Table 1. Cloned DNA fragments derived from the B chromosome

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
N	Clone number	Size (b.p)	AT (%)	Signal on Bs	Signal on As	Comments	Homology to other clones
Group of clones showed homology to each other							
1	#1	233	60.94	WB	PR	NSH	#12 – 77%, #19 – 83%, #37 – 80%
2	#12	216	60.19	PR	PR	NSH	#1 – 77%, #19 – 82%, #37 – 77%
3	#19	291	58.08	WB	PR	NSH	#1 – 83%, #12 – 82%, #37 – 82%
4	#37	334	61.98	WB	ND	NSH	#1 – 80%, #12 – 77%, #19 – 82%
Group of clones showed homology to each other							
5	#6	193	53.89	WB	PR	Part of clone consist of tandem repeat; size of repeat unit is 17 bp; clone showed 69% homology to subtelomeric repeats of *Bombyx mori*	#43 – 93%, #97 – 76%
6	#43	160	50.62	WB	PR	Ditto	#6– 93%, #97% – 76%
7	#97	301	46.18	WB	ND	Ditto	#6 – 79%, #43 – 76%
Group of clones showed homology to each other							
8	#10	365	58.63	PR+DR	PR	NSH	#33 – 91%
9	#33	301	56,88	PR+DR	PR	NSH	#10 – 91%
Group of clones showed homology to each other							
10	#93	244	66,80	PR	PR	Clone showed 76% homology to *Locusta migratoria solitarious* EST clone (ACC CO856777)	#118 – 100%
11	#118	244	66,80	PR	PR	Ditto	#93 – 100%
Clones showed no homology to each other							
12	#23	137	63.50	ND	ND	NSH	
13	#26	272	61,98	ND	ND	Clone showed 98% homology to *H. sapiens* BAC clone RP11-804M4 (acc AC073284)	
14	#29	328	61.59	WB	ER	Clone showed 75% homology to *Locusta migratoria solitarious* EST clone (ACC CO843638)	
15	#85	234	55,13	WB	PR	The tandem repeats with 38bp consensus, NSH	
16	#86	296	66,89	WB	PR	NSH	
17	#87	406	57.88	WB	ND	NSH	

WB, the whole B chromosomes; PR, pericentric regions; PR+DR, pericentric region and distal region of long arm of B chromosomes; ER, euchromatic regions of A chromosomes; ND, no region with detectable specific signal; NSH, no significant homology with known DNA sequences.

FISH signal on this B was similar to the signal in NORs of A chromosomes. The distribution of rDNA in *P. sapporensis* was different. In *P. sapporensis* FISH with rDNA gave a signal in C-positive regions of the Bs of different morphotypes and in additional C-positive short arms of A chromosomes (Bugrov *et al.* 2004) (Figure 3c). No signal was detected in telomeric regions of long arms of the A chromosomes.

Sequencing of cloned DNA fragments and their FISH on A and B chromosomes in P. kanoi

In order to analyze the DNA content of the Bs in *P. kanoi*, 153 DNA fragments derived from the Bk microdissected library were cloned in pBluescript II KS+ (60 clones) and in pCR4Blunt-TOPO vectors (93 clones). The size of cloned DNA fragments ranged from 100 up to 500 bp. A total of 20 cloned DNA fragments were sequenced and computer analysis of their sequences was performed. Three of them showed homology to plasmid DNA and were excluded from further analysis.

Patterns of FISH signal obtained with the remaining 17 clones were divided into six groups: *i* – six clones painted the whole B and gave a signal in A chromosome pericentric regions; *ii* – one clone painted the whole B and gave a slight signal in A chromosome arms; *iii* – three clones painted the whole B and gave no signal in A chromosomes; *iv* – three clones painted the pericentric regions of A and B chromosomes; *v* – two clones hybridized to pericentric regions of A and B chromosomes and subtelomeric regions of the Bs; *vi* – two clones gave only a background-like signal on A and B chromosomes (Table 1). Results of FISH with DNA probes based on five cloned DNA fragments are shown in Figure 4.

Sequences of the clones were submitted to DDBJ/GenBank/EMBL (http://www.ddbj.nig.ac.jp/) (Accession Nos: EF053372–EF053387) and compared with known sequences. 16 of them were AT-enriched. Only one clone (clone #97) was GC-enriched. This clone showed 69% homology to a tandem repeat in *Bombyx mori* (Kang *et al.* 2004). The size of the repeated element in clone #97 was 17bp (TGAA ACCTGACCTGACC). In *P. kanoi* its copies were distributed along the entire B; however, FISH with the probe based on this clone revealed no specific signal on A chromosomes. Nine clones could be combined into three groups according to their homology to each other: *i* – clones #1, #12, #19, and #37; *ii* – clones #6, #43, and #97; *iii* – clones #10 and #33 (Table 1). Clones #93 and 118 were identical. They showed 76% homology to *Locusta migratoria* solitarious phase hind-leg cDNA (EST clone; ACC CO856777). Clone #29 showed 75% homology to *Locusta migratoria* solitarious phase EST clone (ACC CO843638). This clone painted the whole B chromosome and pericentric regions of A chromosomes. DNA probes based on two clones (# 23 and #26) showed no FISH signal on A or B chromosomes. Furthermore, clone #26 showed 98% homology to human BAC RP11-804M4 (ACC AC073284).

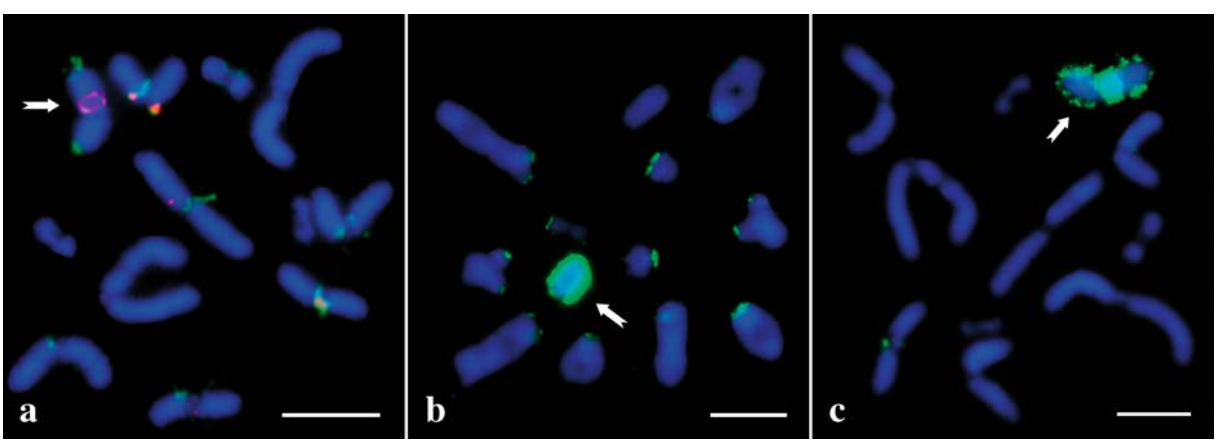

Figure 4. FISH of *P. kanoi* cloned DNA fragments on *P. kanoi* meiotic chromosomes: (**a**) #12 probe (red), #10 probe (green) (metaphase II); (**b**) #23 probe (red), #43 (green) probe (metaphase I); (**c**) – #19 (green) (metaphase II). Counterstained with DAPI. Arrow indicates B chromosome. Scale bars = 10μm.

There are probably only a few copies of DNA elements homologous to these clones, although we cannot exclude contamination as their origin. However, most of the cloned DNA fragments were obviously derived from the B chromosome. Fifteen of 17 DNA probes based on these clones painted the whole B or some of its regions.

Discussion

FISH with DNA probes ranging in size from 100 to 500bp marks chromosome regions only if there are numerous copies of homologous sequences present in the chromosome. The patterns of FISH signal on the Bs obtained with DNA probes based on cloned DNA fragments indicate a wide distribution of numerous copies of repeats in the B in *P. kanoi*. Repeats homologous to cloned DNA fragments were found in the Bs and in pericentric regions of A chromosomes.

Through the analysis of the mechanisms of B chromosome formation we considered the hypothetical evolutionary pathways of chromosome rearrangements and possible B chromosome organization based on the presented data (Figure 5). We assumed that the B chromosome at its initial stage was small, and similar to human small supernumerary marker chromosomes (SMCs), reviewed in detail in Liehr *et al.* (2004). Generally, SMCs are formed as a result of the deletion of chromosome arms followed by telomerization of chromosome regions at the breakpoints or inverted duplication of short arm and pericentromeric regions of acrocentric chromosomes.

In contrast to human SMCs, the size of the initial B chromosome was enlarged through DNA amplification. Indeed, six DNA probes based on cloned

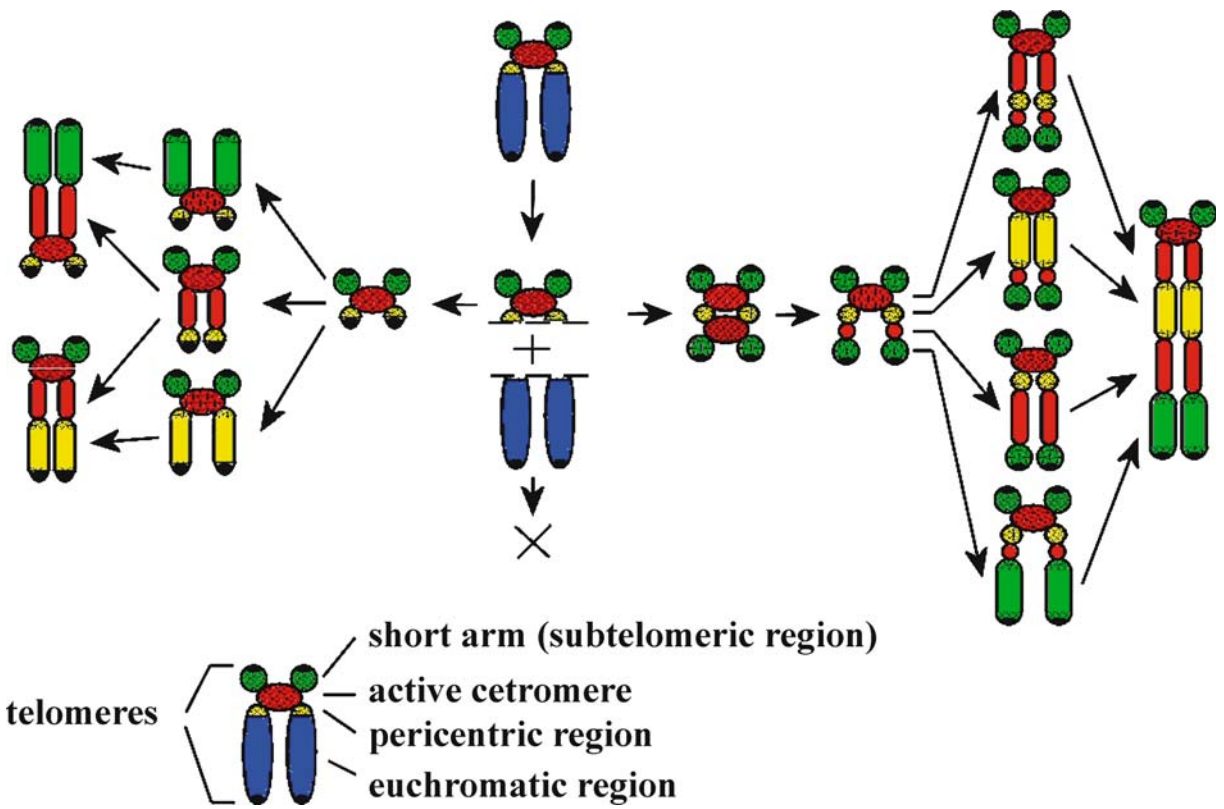

Figure 5. Hypothetical mechanisms of B chromosome formation and evolution in *P. kanoi*. Chromosome regions are described in the figure. Arrows indicate chromosome breaks, formation of inverted duplication, centromere inactivation, chromosome end telomerization, amplification of DNA in chromosome regions. The first break led to small additional chromosome formation as result of deletion of most of the long arm accompanied with inverted duplication or telomerization of the chromosome end. In case of inverted duplication the next step was one centromere inactivation. Further evolution in both hypothetical mechanisms was associated with DNA amplification in different chromosome regions.

DNA fragments painted the whole B and gave a signal on pericentric regions or short arms of A chromosomes. Two other DNA probes gave a signal on pericentric regions including short arms of A and B chromosomes and on subtelomeric regions of the long arm of the B. Three DNA probes marked only the pericentric regions of A and B chromosomes (Table 1). This means that most of the B chromosome DNA was derived from the original A chromosome.

In Figure 5 we summarize the hypothetical pathways of B chromosome evolution by A chromosome rearrangements and DNA amplification in different regions of the initial B chromosome. The formation of the initial B as a ring chromosome was excluded from our analysis. This type of rearrangement requires two simultaneous breaks in both arms of the ancestral chromosome. Furthermore, in order to form the modern morphology of the B, one more break in the ring chromosome followed by telomerization of chromosome regions at the breakpoints is required. This sequence of events is considered to occur rarely, if ever. It is also unlikely that contemporary Bs are derived from the deletion of the ancestral chromosome arm followed by telomerization of regions at the breakpoint and DNA amplification because of the existence of two DNA probes (#10 and #33) that produced a signal on pericentric regions, including the short arm, and on subtelomeric regions of the long arm of B chromosomes (Figure 4a, Table 1). Therefore, the most promising hypothesis that agrees well with the present results is that the initial B chromosome is an inverted duplication of the short arm and pericentromeric region of an acrocentric chromosome followed by an inactivation of one centromere. A similar mechanism was shown for formation of human small SMCs from acrocentric chromosomes. Moreover, as shown by the location of FISH signals, DNA fragments from different regions have been amplified in the B. Different regions of A and B chromosomes were marked by probes based on cloned DNA fragments derived from the B chromosome. In all cases FISH of these probes painted large regions on B chromosomes, implying independent DNA amplification in different regions. Therefore, all regions of the initial B were possibly amplified during B chromosome evolution. Despite only 17 cloned DNA fragments in FISH experiments, these probes painted various regions of chromosomes, e.g. only the pericentromeric region, pericentromeric and subtelomeric regions, the whole B chromosomes, and slightly euchromatic regions of A chromosomes.

Unexpectedly, DNA clones that marked relatively small B chromosome regions were overrepresented. Three of 17 clones marked only the pericentric region and two produced a signal in both pericentric and subtelomeric regions. These regions cover a small part of the entire B, and the DNA clones derived from these regions should comprise a small portion of the B chromosome DNA library. B chromosome probe #19 helps to resolve this contradiction (Figure 4c). This probe painted the entire B chromosome; however, signal intensity was much stronger in the pericentric region than in the long chromosome arm. Some copies of other pericentric-specific DNA sequences were probably also present in the long chromosome arm as dispersed repeats.

It is uncertain whether all of the contemporary B chromosome DNA is derived from the initial B or partially from inserted DNA elements. In humans the phenomenon of duplicons associated with DNA transposition has been described and molecular mechanisms involved in duplicon formation were discussed by Sharp *et al*. (2005) and Bailey *et al*. (2003). Insertion of DNA elements into the B chromosome could have taken place by similar mechanisms. DNA probe #29 painted the whole B but gave only a very weak, dispersed signal on euchromatic A chromosome regions. Other DNA probes (#37, #87, #97) also painted the whole B but gave no signal on the A chromosomes. Unfortunately we could not ascertain the origin of these DNA fragments.

Our present results strongly suggest that at least some DNA transpositions into the B chromosomes occurred after formation from the ancestral B. The rDNA cluster found in one of the studied Bs may be the result of rDNA transposition during the later stage of B chromosome evolution. Regions enriched with rDNA were found in B chromosomes in many other species (Green 1990). In general there are two forms of rDNA distribution in B chromosomes including large regions enriched with rDNA in the B chromosomes of some species (Cabrero *et al*. 2003), and clusters of rDNA shown to be small NORs in others (Rubtsov *et al*. 2004). It should be noted that almost all B chromosomes in *P. sapporensis* contained large regions enriched with rDNA. In

contrast to *P. kanoi*, rDNA in A chromosomes in *P. sapporensis* is located in pericentric regions and in additional short arms. The ancestor of the B chromosomes in *P. sapporensis* probably had a cluster of rDNA in its pericentric region or short arm and rDNA was included into the initial B as a result of chromosome rearrangement that led to inverted duplication of the short arm and proximal part of the long arm. In *P. kanoi*, NORs were located in subtelomeric regions of long arms of the X and the middle-sized acrocentric autosome. As suggested, formation of the supernumerary chromosome in *P. kanoi* would not lead to the inclusion of rDNA in the initial B chromosome. Indeed, rDNA was atypical for the Bs in *P. kanoi*. The rDNA cluster found in the only B chromosome could have originated from a standard NOR of an A chromosome. Transposition of rDNA could have occurred by means of alternate telomere lengthening, the mechanisms of which include non-homologous recombination of subtelomeric regions (Londono- Vallejo *et al*. 2004, Laud *et al*. 2005). It is likely that the large difference in molecular composition between *P. kanoi* and *P. sapporensis* Bs is due to the different patterns of rDNA distribution in A chromosomes in these. While B chromosomes in *P. sapporensis* included spacious regions enriched with rDNA, a small cluster of rDNA was found only in one B chromosome of *P. kanoi*.

Generation of the B chromosome DNA library, cloning of B chromosome DNA fragments, and comparative FISH analysis showed that the B chromosomes in *P. kanoi* consist mainly of repetitive DNA. A detailed study of DNA composition and evolution of the B chromosomes in *P. kanoi* through comparative analysis of DNA sequences revealed that the sequenced DNA fragments were mostly AT-rich and some of them consisted of short repetitive units (Table 1).

Short repeated sequences were also found in short cloned DNA fragments. Their size in clones #6, #43, and #97 was equal to 17bp. In clone #97 this repeat showed differences in two positions. In clones #6 and #43 repeat units were identical. The presence of similar repeats in these clones led to the high homology between them (Table 1). A high level of homology was revealed between these clones and DNA of subtelomeric regions in *Bombix mori* chromosomes. Substantial similarity between clones # 10 and #33 was also disclosed (91% homology), as well as for the group of clones #1, #12, #19 and #37 (Table 1). Another group (clones #29, #93, #118) showed homology with different EST clones extracted from *L. migratoria*. The identical clones #93 and #118 are probably derived from the same fragment that was amplified during DNA library generation. The rest of the sequenced DNA fragments did not show significant homology with known sequences.

FISH of different DNA probes gave different painting patterns in pericentromeric regions and short arms of A chromosomes. Some marked pericentromeric regions of all chromosomes, while others gave a signal in pericentromeric regions only in a few chromosomes. These data show that there are DNA sequences that are common to all pericentromeric regions, while the distribution of other sequences in pericentromeric regions is limited to one or a few pairs of A chromosomes. Some clusters of repeat may generate a hot break spot in one of the autosomes, increasing the frequency of B chromosomes formation.

In conclusion we have to mention that *P. sapporensis* and *P. kanoi* are very closely related species. Results of comparative molecular cytogenetic analysis of A and B chromosomes performed in this study showed that relatively small chromosome reorganization such as relocation of NORs from pericentromeric regions into telomeric regions or, on the contrary, could have a significant influence on further karyotype evolution in these species. As for B chromosomes in *P. sapporensis* they are enriched with rDNA that was included in the Bs, probably since the moment of their formation. In *P. kanoi* the Bs, had probably no rDNA in the beginning and their evolution was associated mainly with amplification of different repeated sequences. We suppose that in animals the frequency of B chromosome formation and their further evolution strongly depends on some traits of molecular and morphological chromosome organization, for example on 'hot spot' in pericentromeric regions of acrocentric chromosomes, different types of repeats distribution, and NOR location.

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