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Comparative analysis of micro and macro B chromosomes in the Korean field mouse *Apodemus peninsulae* (Rodentia, Murinae) performed by chromosome microdissection and FISH

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Abstract. Comparative analysis of micro B and macro B chromosomes of the Korean field mouse *Apodemus peninsulae*, collected in populations from Siberia and the Russian Far East, was performed with Giemsa, DAPI, Ag-NOR staining and chromosome painting with whole and partial chromosome probes generated by microdissection and DOP-PCR. DNA composition of micro B chromosomes was different from that of macro B chromosomes. All analyzed micro B chromosomes contained clusters of DNA repeats associated with regions characterized by an uncondensed state in mitosis. Giemsa and DAPI staining did not reveal these regions. Their presence in micro B chromosomes led to their special morphology and underestimation in size. DNA repeat clusters homologous to DNA of micro B chromosome arms were also revealed in telomeric regions of some macro B chromosomes of specimens captured in Siberian regions. Neither active NORs nor clusters of ribosomal DNA were found in the uncondensed regions of micro B chromosomes. Possible evolutionary pathways for the origin of macro and micro B chromosomes are discussed.

Extensive cytological studies of B chromosomes in the Korean field mouse (*Apodemus peninsulae* Thom.) have been performed for many years. Thousands of animals from populations distributed around a huge area including Western Siberia, North Mongolia, North East China, the Russian Far East, Korea and Japan (Hokkaido) have been karyotyped (for review, see Kartavtseva and Roslik, this issue). According to their morphology detected by Giemsa and DAPI staining, Bs were divided into four groups: (1) large metacentrics or submetacentrics; (2) medium-to-small metacentrics or submetacentrics; (3) medium-to-small acrocentrics or subtelocentrics; and (4) dot-like (micro B) chromosomes (Kartavtseva et al., 2000). Extensive intraindividual, intrapopulational, and interpopulational variability for B chromosome number and morphology have been found. Recently, repeated DNA of Bs in the Korean field mouse has been analyzed by FISH with DNA probes generated by microdissection of A and B chromosomes followed by DOP-PCR (Karamysheva et al., 2002). It was shown that all B chromosomes were composed of a large amount of repeated DNA sequences. The repeats were classified in terms of their homology and predominant location. Pericentromeric repeats of the Bs were homologous to repetitive DNA of pericentromeric C-blocks of all autosomes and non-centromeric C-blocks of the sex chromosomes. Two other types of repeats comprised the main body of the arms of the majority of the Bs. One type of B

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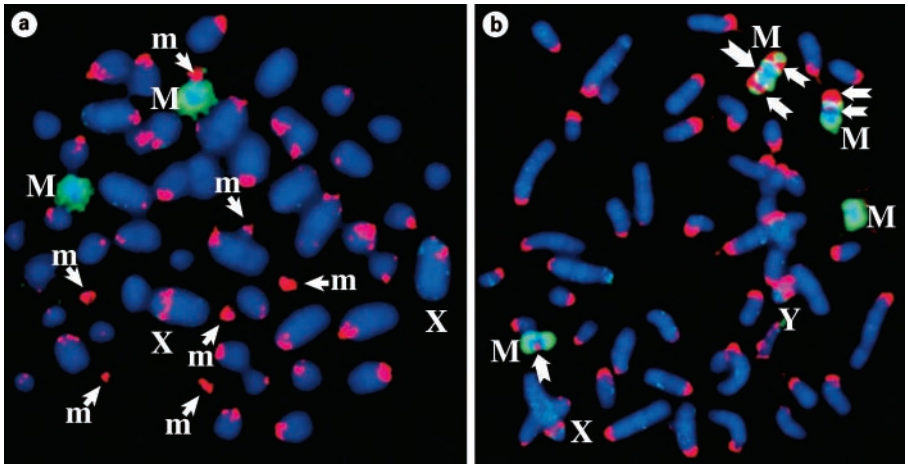

Fig. 1. Two-color FISH with metaphase chromosomes of *A. peninsulae* with the DNA probes derived from the arms of macro B chromosomes (green) and pericentromeric C-positive autosome region (red); DAPI counterstaining is blue; micro Bs are marked with **m** and indicated with arrows (**a**); macro Bs are marked with **M** (**a**, **b**); sex chromosomes are marked with **X** and **Y**; arrows with swallowtail point to macro B chromosome pericentromeric, intercalary, and telomeric regions containing DNA homologous to DNA of autosome pericentromeric regions (**b**).

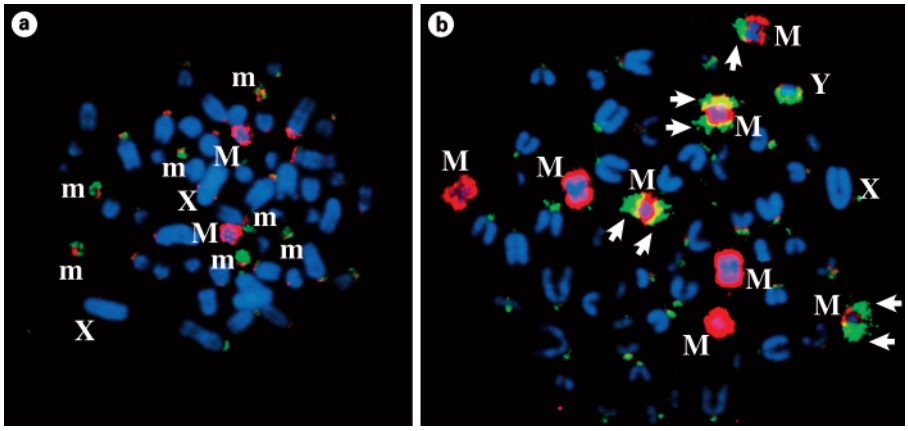

Fig. 2. Two-color FISH with metaphase chromosomes of *A. peninsulae* with the DNA probes derived from the micro B (green) and the small Siberian macro B (red); DAPI counterstaining is blue; metaphase spread with micro B chromosomes (**a**); metaphase spread with macro B chromosomes containing uncondensed telomeric regions (**b**); micro Bs are marked with **m**; macro Bs are marked with **M**; arrows indicate uncondensed regions in macro Bs; sex chromosomes are marked with X and Y.

chromosome arm repeats also showed homology to repeated interspersed sequences in euchromatic regions of the As (Karamysheva et al., 2002). The second type of DNA repeats was not detected by FISH in the A chromosomes.

Distribution of these types of repetitive DNA sequences was also analyzed in the closely related species, *A. agrarius.* By comparing the distribution of these DNA repeats in the chromosomes of the two species, *A. peninsulae* and *A. agrarius,* an assumption of B chromosome origin and evolution in the Korean field mouse was suggested. It implied that DNA sequences which persisted in euchromatic parts of A chromosomes under stringent control of natural selection invaded pericentromeric regions. At the new position, they were involved in amplification resulting in destabilization of pericentromeric regions. This led to a high frequency of micro chromosome formation (Karamysheva et al., 2002). Similar supernumerary familial marker chromosomes containing no euchromatic region and showing no apparent phenotypic effects are well known in human cytogenetics (see Fuster et al., this issue). When the small proto-B chromosomes were established in this way, different types of A chromosomal or extrachromosomal DNA sequences might have invaded them, were amplified, and led to the Bs of a larger size. Very small Bs (dot-like or micro Bs) have been found in many populations of the Korean field mouse. They were considered to be B chromosomes in the initial stage of their development. Their number varied from 0 to 20 per specimen (Kartavtseva, 1999; Kartavtseva and Roslik, this issue). In this study, we analyze DNA composition of these micro B chromosomes and try to ascertain the reason for inter-population differences in their frequency.

Materials and methods

Overall, 51 specimens of *A. peninsulae* captured in Siberia (31 specimens) and the Russian Far East (20 specimens) were studied. Chromosomes for routine and FISH analysis were prepared from short-term bone marrow cell cultures. DAPI, Giemsa, and Ag-NOR staining was performed according to standard protocols (Verma and Babu, 1995).

B chromosomes were characterized by FISH with DNA probes generated by microdissection of metaphase chromosomes followed by DOP-PCR with the MW6 primer, and subsequent DNA labeling with biotin-16-dUTP or digoxigenin-11-dUTP performed in 15 additional PCR cycles (Rubtsov et al., 2000). DNA probes derived from autosome pericentromeric C-positive regions, arms of macro B chromosomes, small macro B chromosomes, and micro B chromosomes derived from Siberian specimens were described earlier (Karamysheva et al., 2002). Five DNA probes derived from five macro B chromosomes found in three specimens captured in the Russian Far East were generated in this study. One of these Bs was small. But it was nearly twice as large as the micro Bs from Siberian specimens. The DNA probe used for detection of clusters of 18S rDNA (rDNA probe) contained a 3.2-kb frag-

ment of human 18S rDNA in pHr13 (Malygin et al., 1992). It was labeled with biotin-16-dUTP by standard nick translation.

FISH was performed according to a standard protocol (Lichter et al., 1988) with salmon sperm DNA as a carrier DNA. Biotin- and digoxigenin-labeled probes were visualized with avidin-FITC and mouse antidigoxigenin antibodies conjugated to Cy3, respectively. Chromosomes were counterstained with DAPI and analyzed using an Axioskop 2 (Zeiss) microscope equipped with a CCD camera, filter set, and ISIS3 image-processing package of Metasystems GmbH.

Results

The number of standard (A) chromosomes was 2n = 46 + XY). In addition, 49 out of the 51 individuals analysed carried 1–10 B chromosomes. The total number of Bs analyzed in the 49 individuals was 215. According to their morphology analyzed by Giemsa and DAPI staining, the Bs were divided into two groups: (1) macro Bs (large-to-small metacentrics, submetacentrics, acrocentrics, and subtelocentrics) and (2) micro Bs (dot-like chromosomes). A very small sized B was considered a micro B whenever it showed no detectable chromosome arm in all metaphase spreads analyzed. On this basis, 21 Bs found in nine animals trapped in the Siberian regions were diagnosed as micro Bs. The highest number of micro Bs in an individual was eight. No micro B was found in specimens captured in the Russian Far East.

A and B chromosomes in all specimens were analyzed by two-color FISH with pairs of the DNA probes mentioned above. The DNA probe derived from autosome pericentromeric C-positive regions painted the pericentromeric regions of autosomes, an intercalary C-block in the X and a telomeric C-block in the Y (Fig. 1a, b). Pericentromeric regions of the X and the Y showed no signal. In Siberian Bs this DNA probe painted pericentromeric regions of all micro Bs and some macro Bs (Fig. 1a, b). In a few macro B chromosomes, this probe also painted intercalary or telomeric regions of arms (Fig. 1b). The size of pericentromeric regions painted on B chromosomes was considerably (5–10 fold) smaller than that in the large autosomes. No signal was detected after FISH with DNA probe derived from autosome pericentromeric C-positive regions on B chromosomes of specimens collected in the Russian Far East. Probes derived from Bs of specimens from the Russian Far East gave no signal in C-positive regions of A chromosomes.

DNA probes derived from arms of Siberian macro B chromosomes painted normally condensed regions of macro B chromosome arms but gave signal neither in the micro Bs nor in C-positive regions of A chromosomes (Fig. 1a, b). All DNA probes derived from the whole B chromosomes of the specimens captured in the Russian Far East painted the same regions that were painted with the DNA probe derived from the arms of the Siberian B chromosome. Additionally DNA probes derived from the Russian Far East Bs painted pericentromeric regions of the Bs in the Russian Far East specimens and pericentromeric regions of some Bs in Siberian specimens.

The DNA probe derived from the small macro B chromosome of the specimen captured in West Siberia painted all regions that showed signal after FISH with the DNA probe derived from autosome pericentromeric C-positive regions and DNA probe derived from arms of the macro B chromosome (Fig. 2a, b). The DNA probe derived from micro B chromosome painted all chromosome regions showing signal after FISH with DNA probe derived from autosome pericentromeric regions (Fig. 2a, b). This probe also painted all micro Bs (Fig. 2a) and telomeric regions of macro Bs showing uncondensed state in mitosis (Figs. 2b, 3). Data obtained with FISH with DNA probes generated by microdissection and DOP-PCR are summarized in Table 1.

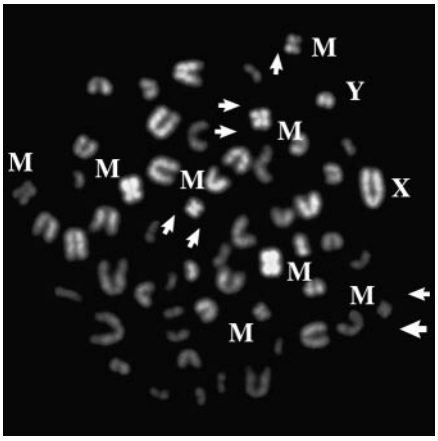

Fig. 3. DAPI staining of the metaphase spread shown on Fig. 2b; arrows indicate location of uncondensed telomeric regions of macro B chromosomes; arrows indicate macro B chromosomes; sex chromosomes are marked with X and Y.

Based on results of FISH experiments, the following types of B chromosome organization were revealed. Micro B chromosomes consisted of two types of regions: (a) small pericentromeric region containing repeats homologous to repeats of A chromosome C-positive regions and (b) region(s) showing an uncondensed state in mitosis and containing specific DNA repeats. Macro Bs could be divided into two groups depending on whether their pericentromeric regions showed homology (group 1) or not (group 2) to C-positive autosome pericentromeric regions. Most macro Bs found in specimens collected in Western Siberia and some macro Bs of specimens from East Siberia belonged to group 1. Other macro Bs of specimens from East Siberia and all Bs from the Russian Far East belonged to group 2. The arms of all macro Bs in both groups contained normally condensed regions enriched with homologous repeats. Some macro Bs in Siberian specimens carried uncondensed DNA regions homologous to those in micro Bs (Fig. 2b).

These uncondensed regions enriched with specific repeats were present in all micro Bs. FISH analysis did not reveal these repeats in A and macro B chromosomes of specimens captured in the Russian Far East. The registered frequency of micro B chromosomes in specimens from this region is very low (Kartavtseva and Roslik, this issue). Furthermore, a detailed analysis of micro Bs from the Russian Far East showed that they were twice as large as the micro Bs from Siberia and showed

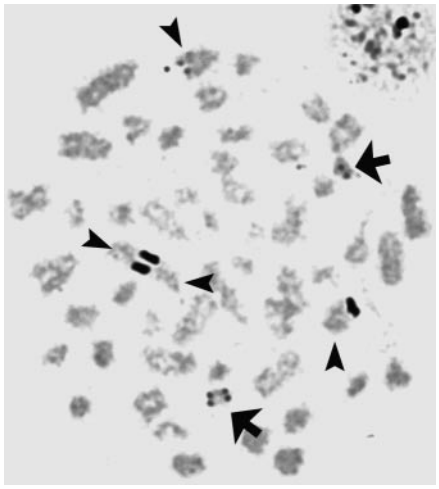

Fig. 4. Ag-NOR banding of Korean field mouse chromosomes; arrows indicate active NORs of the macro Bs; arrowheads indicate autosomes with active NORs.

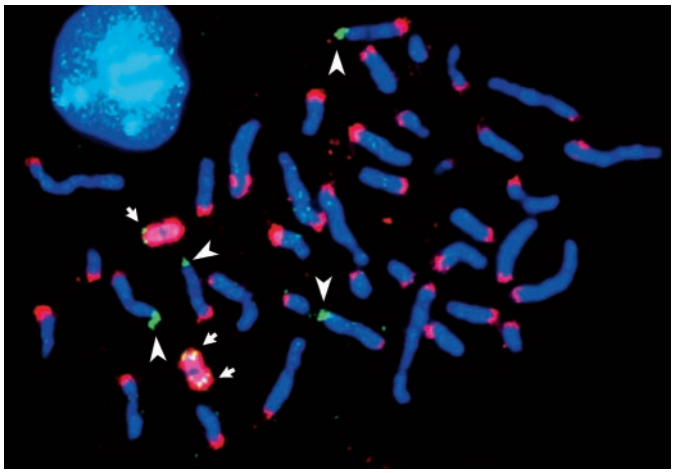

Fig. 5. Two-color FISH with metaphase chromosomes of *A. peninsulae* with the DNA probes derived from the small Siberian macro B (red) and rDNA probe (green); arrows indicate NORs of the macro Bs; arrowheads indicate NORs of autosomes.

Table 1. Painting patterns[a] of the A and B chromosomes produced by FISH with microdissected DNA probes

Probe derived from	A chromosomes		Macro Bs					Micro Bs		Figures
	C-blocks	Euchromatic regions	Siberian Bs			Russian Far East Bs		Pericentromeric regions	Uncondensed region	
			Pericentromeric regions	Arms	Uncondensed regions	Pericentromeric regions	Arms			
Autosome pericentromeric region	+++	–	+–	–	–	–	–	++	–	1a, 1b
Arms of macro Bs	–	+	–	+++	–	–	+++	–	–	1a, 1b
Small macro B from Siberia	++	+	+–	+++	–	–	+++	++	–	2, 3
Large macro B from the RFE[b]	–	+	+–	+++	–	+	+++	–	–	NS[c]
Small macro B from the RFE[b]	–	+	+–	+++	–	+	+++	–	–	NS[c]
Micro B	++	–	+–	–	+++	–	–	++	+++	1, 2, 3, 4

[a] Regions are painted heavily (+++), with middle intensity (++), a little more intensive than the background (+), as background (–); regions of some chromosomes showed signal whereas regions of other chromosomes showed no signal (+–).
[b] The Russian Far East (RFE).
[c] Results that were not shown on the pictures (NS).

small arms in good quality metaphase spreads. According to their size and morphology they should be considered as small macro B chromosomes, rather than micro Bs. One of these B chromosomes was analyzed by FISH with all available DNA probes. It was painted with DNA probes derived from macro Bs but showed no signal after FISH with DNA probes derived from micro B chromosomes. Conversely, the DNA probe derived from this small B chromosome produced painting patterns in A and B chromosomes identical to those obtained with other DNA probes derived from macro Bs from the Russian Far East (Table 1). These data showed that DNA composition in this small macro B chromosome is similar to that of other macro Bs from the Russian Far East.

In an attempt to discover the nature of the uncondensed DNA regions in micro B chromosomes, we analyzed the rDNA location. rDNA is one of the types of tandemly repeated DNA, which has been frequently revealed in B chromosomes in many species (Green, 1990; Jones, 1995). Active nucleolar organizing regions (NOR) remain usually in an uncondensed state in mammalian mitotic chromosomes. Active NORs and clusters of rDNA in the As and Bs of the Korean field mouse were detected with Ag-NOR banding and FISH with the rDNA probe. The Ag-NOR banding heavily stained the telomeric regions of three or four autosomes (two pairs of middle-sized acrocentrics). Small active NORs in telomeric or intercalary regions of one or both arms of 28 macro Bs in the 24 animals from Siberia and the Russian Far East were additionally revealed (Fig. 4). Ag-positive regions in the Bs were usually similar in size to the smallest Ag-positive regions in the As. Only one B chromosome showed an active NOR comparable in

size with large NORs of autosomes. FISH with the rDNA probe gave signals at the Ag-NOR positive regions of the As and Bs. In the Bs, they were usually smaller than in autosomes (Fig. 5). No micro Bs showed active NORs or clusters of rDNA detectable by the techniques used in this study. Furthermore, all rDNA clusters in the macro B chromosomes were located in regions containing repeats characteristic for normally condensed arms of macro B chromosomes. Consideration of these results led to the conclusion that the uncondensed state of the micro B chromosomes was not associated with the location of active NORs.

Discussion

A variety of hypotheses suggesting mechanisms for the evolutionary origin and further development of B chromosomes have been proposed and discussed (for review see Camacho et al., 2000). The most widely accepted view is that they are derived from A chromosomes. For instance, they could derive from the leftover centromere from A chromosome fusions (Patton, 1977), from polysomic chromosomes, from amplified pericentromeric chromosome fragments (Keyl and Hagele, 1971), or they could arise from fragments formed in trisomic pairing (Amos and Dover, 1981). Apart from the many Bs of autosomal origin (Peppers et al., 1997), sex chromosome-derived Bs have also been described (Sharbel et al., 1998; Cabrero et al., 2003). This view is based on the observation that B chromosomes display a certain degree of homology with some regions of A chromosomes (Jamilena et al., 1994, 1995; McQuade et al., 1994; Stark et al., 1996). There are also B chromosomes that are supposed to be composed of transposable elements (McAllister, 1995; Peppers et al., 1997).

Data on DNA homology of B chromosome pericentromeric regions to autosome pericentromeric regions in *A. peninsulae* (Karamysheva et al., 2002) suggested the autosomal origin of some Bs in this species. However further investigation involving B chromosomes from the Russian Far East population revealed the existence of Bs that did not contain DNA homologous to DNA of autosome pericentromeric regions (Table 1). They probably originated from sex chromosomes whose pericentromeric regions showed no homology to autosome pericentromeric regions. Multiregional origin of B chromosomes has recently been shown in the grasshopper *Eyprepocnemis plorans* (Cabrero et al., 2003). The available data in the Korean field mouse also suggest this possibility.

A possible pathway for B chromosome origin could resemble the mechanism suggested by Wandstrat and Schwartz (2000) for the origin of some human supernumerary marker chromosomes (SMC). It would involve illegitimate recombination between homologous acrocentrics followed by nondisjunction and centromere inactivation, with breakpoints localized within the array of DNA repeats in heterochromatic regions or else in euchromatic regions containing several low-copy repeat DNA sequence families. One break and erroneous reparation event leads to the formation of an inverted duplication. In fact, most of the SMCs derived from acrocentrics are inv dup(15). The different frequency of SMCs derived from human acrocentrics presumably indicates the different incidence of chromosome rearrangements in their pericentromeric regions. The small size of B chromosome pericentromeric regions in *A. peninsulae*, which are homologous to autosome pericentromeric regions, leads to the suggestion that at least one breakpoint located within the autosome C-heterochromatic pericentromeric region might have been involved in the formation of these Bs. The suggestion that the formation of the proto-B chromosome could go through a stage of inverted duplication might explain the presence of telomeric repeat clusters in all analyzed Bs in *A. peninsulae* (Karamysheva et al., 2002). DNA amplification during B chromosome development should lead to difference in size and DNA composition of B chromosome arms. However many large Bs looked like isochromosomes (Karamysheva et al., 2002). This phenomenon could be the consequence of high rate of B chromosome rearrangements with hot spots in their pericentromeric regions forming isochromosomes in the late stages of B chromosome evolution.

Presence of NORs in telomeric regions of some B chromosomes could suggest another possible mechanism for B chromosome formation. B chromosomes could derive from autosomes by deletion with one breakpoint in pericentromeric regions and another breakpoint near the telomere. This suggestion can explain the existence of B chromosomes with NORs in telomeric position. At least some of them could derive from autosomes containing NORs by deletion of a large chromosome region. However, insertion of rDNA into B chromosomes could take place on the late stage of their evolution. The latter suggestion could explain NOR locations in Bs derived from the Russian Far East population. The origin of these Bs was discussed above. They were probably derived from sex chromosomes, which contained neither detectable active NORs nor clusters of rDNA.

The consideration of possible pathways for B chromosome origin in *A. peninsulae* has led us to the conclusion that the proto-B chromosome(s) should be small and contain regions originating from A chromosomes. Further proto-B evolution to become Bs as those found in current natural populations would probably be based on the insertion and amplification of new DNA elements. The final B size and morphology would depend on the type of DNA elements inserted and the intensity of their amplification. Insertion and amplification of DNA elements is a common feature of B chromosome evolution in many species (for review see Camacho et al., 2000). But a peculiarity in the development of some B chromosomes was revealed in Siberian populations of *A. peninsulae*. While most proto-Bs developed into macro Bs, others acquired regions which remain uncondensed in mitosis. In these Bs only a small pericentromeric region could be visualized in metaphase spreads with Giemsa or DAPI staining. According to their small size and special morphology they were named micro Bs. It is conceivable that some micro Bs evolve into macro Bs through the insertion and amplification of certain DNA elements, in which case we should expect that they contain uncondensed DNA regions similar to those in micro Bs. This was the case in some specimens from Siberia. Alternatively, they could arise by insertion or translocation of micro B uncondensed DNA repeats into telomeric regions of macro B chromosomes. Furthermore, the probability that all DNA sequences found in B chromosomes

were already present in the proto-B or even in the ancestor A chromosome cannot be ruled out. In this case, differential amplification of these existing sequences would explain the different B chromosome types found.

The origin of DNA repeats in the uncondensed regions of B chromosomes remains unexplained. We know that they are not rDNA, and the question of their possible transcriptional activity remains open. FISH revealed regions enriched with these repeats only in B chromosomes of specimens from Siberia and they were not detected in specimens from the Russian Far East. Perhaps a multiregional origin of Bs might explain these differences, but testing this suggestion requires further investigation including cloning and sequencing.

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