ANIMAL
GENETICS

Clusters of Repetitive DNA Sequences in Chromosomes of Voles of the Subgenus *Microtus* (*Microtus*, Arvicolidae)

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Abstract—The study is focused on the analysis of the mechanisms underlying the formation and distribution of repeat clusters in mammalian chromosomes, as exemplified by a group of closely related species of voles of the subgenus *Microtus* (*Microtus*, Arvicolini). The distribution of repetitive sequences that are the parts of *de novo* formed heterochromatic regions of *Microtus arvalis* in two chromosomal forms of this species and three closely related species, *M. rossiaemeridionalis*, *M. kirgisorum*, and *M. transcaspicus*, was analyzed in detail. Possible relationships between the introduction of repetitive sequences, their transpositions, amplification, and the formation of reproductive isolation, which can lead to speciation, are discussed.

Keywords: genome and karyotype evolution, voles of the subgenus *Microtus*, repetitive sequences, speciation, microdissection of metaphase chromosomes, FISH

INTRODUCTION

Constitutive heterochromatin regions detected in mammalian chromosomes, such as C-positive regions of chromosomes, represent a highly variable part of the genome. They demonstrate high variability in localization, size, and even DNA composition both in closely related species and within the same species [1–3]. In addition to large C-positive regions, in mammalian chromosomes, the regions enriched in repetitive sequences but not detected by differential C-banding were described [4]. These regions differ in size, number, and type of repetitive sequences. It was demonstrated that they could be chromosomal segments that were most often involved in evolutionarily important chromosomal rearrangements [4–6]. However, the patterns of formation of such regions and their further evolution remain poorly studied. One of the possible approaches to determine the localization patterns of repeats clusters or regions enriched in repeats in mammalian chromosomes is to obtain microdissected DNA probes from C-positive regions of chromosomes and to determine the distribution of repetitive sequences contained in them both in chromosomes of the original species and in chromosomes of closely related species [1, 7]. Of particular interest in such studies is the analysis of the chromosome distribution patterns of repetitive sequences that are parts of the newly emerging C-positive regions. These regions include C-positive regions of chromosomes which are present only in a small number of individuals of the studied species.

Two such chromosomal regions were previously found in chromosomes 5 and 8 of *Microtus arvalis*, chromosomal form obscurus (hereinafter *M. arvalis* obscurus) [4, 8]. The new variant of chromosome 5 (hereinafter chromosome 5^R) was almost an acrocentric chromosome with a large block of pericentromeric heterochromatin (Fig. 1), small short arms, and active nucleolus organizer regions (NORs), located in distal parts of these short arms. The appearance of the C-positive heterochromatic block in chromosome 5 was accompanied by a change in the centromere position and the appearance of active NOR. The identified derivative of the *M. arvalis* obscurus chromosome 8 (hereinafter chromosome 8^R) was considerably larger than the original chromosome 8 because of the additional C-positive region in the distal part of the long arm, in which active NORs were also detected (Fig. 1) This did not result in a visible change in the position of the centromeric region [4]. It should be noted that, in chromosomes 5 and 8 of *M. arvalis* obscurus, carrying no large additional heterochromatic blocks, no active NORs were detected in any case.

The chromosomes of voles from the group *arvalis* of the subgenus *Microtus* have long been the subject of intensive studies. The rearranged acrocentric chromosome 5^R (as a rule, one homolog in a pair) was found in individual voles in different natural populations
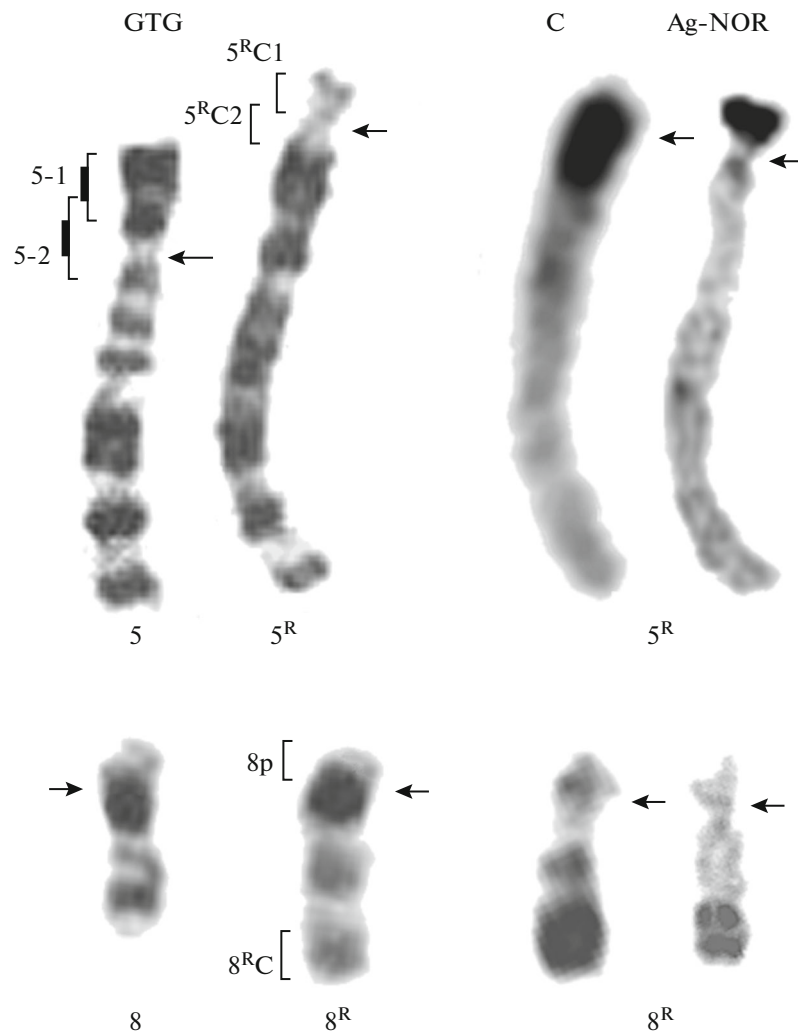
Fig. 1. Chromosomes 5 and 8 of the karyotype of *M. arvalis* obscurus and their reorganized homologs (5^R and 8^R). Differentially stained chromosomes are presented (GTG banding; C banding, Ag-NOR staining). The dissection regions upon production of the DNA probes are indicated by the brackets. The regions with maximum number of collected copies that were used to generate the DNA probes are indicated by thickened lines. Arrows point to the centromeric regions of chromosomes.

from the Volga region, Urals, Novosibirsk oblast, Altai, Armenia, Kazakhstan, and Azerbaijan. In one of the Armenian populations, the frequency of individuals with this C-heterochromatic block reached 10% [8–10]. At the moment, only a single case of the presence of the additional C-positive region in the distal part of the long arm of one of the homologs of *M. arvalis* obscurus chromosome 8 is described. The chromosome 8^R was found in an individual vole captured on the territory of Armenia [4].

Some researchers previously interpreted the appearance of chromosome 5^R in *M. arvalis* obscurus as a result of inversion of the short arm of chromosome 5 with subsequent amplification of pericentromeric DNA, which led to a change in the centromere position and the formation of the C-positive heterochromatic block. However, comparative analysis of differential GTG banding of chromosomes 5 and 5^R did not allow the researchers to come to a final conclusion on this issue [8, 10].

In the present study, a detailed comparison of the organization of the short arm of chromosome 5 and the region of chromosome 5^R containing homologous DNA sequences was performed. The distribution of repeat clusters homologous to repeated DNA sequences of unusual C-positive regions of chromosomes 5^R and 8^R in chromosomes of closely related species of the *arvalis* group of the genus *Microtus* was also analyzed.

MATERIALS AND METHODS

Metaphase chromosome spreads: preparation, staining, description. Vole metaphase chromosome spreads were prepared from previously obtained primary lung

fibroblast cultures of *M. arvalis* (chromosomal forms arvalis and obscurus), *Microtus rossiaemeridionalis*, *Microtus kirgisorum*, and *Microtus transcaspicus* [4] as described earlier [11]. Chromosome staining with Giemsa (GTG staining), DAPI, and Ag-NOR staining was performed according to standard protocols. The preparations were described using generally accepted chromosome nomenclature [9].

Generation of microdissected DNA probes for multi-color banding. Microdissection of the chromosomal regions was carried out in such a way that the number of copies of the collected chromosomal fragments varied, decreasing in the direction of the dissection region boundary. The dissection regions were determined with overlap. Generation of DNA probes from the dissected material was carried out according to the standard protocol [12, 13].

Region-specific DNA probes. Fluorescent *in situ* hybridization (FISH) was performed using previously obtained region-specific microdissected DNA probes from ordinary chromosomes 5 and 8, as well as from the C-positive regions of the rearranged chromosomes 5^R and 8^R of *M. arvalis* obscurus [4]. These DNA probes were obtained in a standard way, including microdissection of the regions of the corresponding chromosomes and subsequent amplification of their DNA in a polymerase chain reaction with partly degenerate primer (DOP-PCR) [12]. The schemes of chromosome dissection are shown in Fig. 1.

Fluorescence in situ hybridization. FISH of region-specific DNA probes with metaphase chromosomes and signal detection (avidin-FITC/biotinylated anti-avidin/avidin-FITC and anti-digoxigenin-Cy3) was performed according to standard methods [12]. For the chromosome staining after *in situ* hybridization, DAPI was used.

Microscopic analysis and treatment of microscopic images. The results of differential staining and FISH were analyzed using an AXIOSKOP 2 Plus microscope (ZEISS, Germany). Microimages were captured using a CCD camera, appropriate Chroma filter sets, and the AxioVision (ZEISS, Germany) and treated by ISIS3 (METASystems GmbH, Germany) software programs.

RESULTS

New C-Positive Region and Centromeric Transposition in Chromosome 5^R of M. arvalis

Chromosome 5^R contained a large C-positive region. The localization of centromere in the center of C-positive block determined the presence in the chromosome of a small C-positive arm. Ag-NOR staining revealed active NOR in its distal part (Fig. 1). To analyze the distribution patterns of repetitive sequences that are part of the de novo formed heterochromatic region in vole chromosomes of the *arvalis* group, FISH with DNA probes obtained by microdissection of de novo heterochromatic regions localized in the

long and short arms of the chromosome were used [4]. The 5^RC1 DNA probe was obtained from the short arm of chromosome 5^R, and the 5^RC2 DNA probe was obtained from the C-positive region localized in the long arm of chromosome 5^R (Fig. 1). FISH of the 5^RC1 DNA probe gave an intensive signal covering the entire C-positive region of chromosome 5^R, but on chromosome 5, it did not produce a visible signal (Fig. 2). Chromosomal in situ suppression hybridization (CISS hybridization) of the 5^RC2 DNA probe intensely painted a part of C-positive region localized in the long arm of chromosome 5^R and less intensely painted a part of C-block localized in its short arm. In addition, this probe painted a fragment of the chromosome 5^R C-negative region, adjacent to the heterochromatic block, and on chromosome 5, it painted the distal part of the short arm (Fig. 2). The CISS hybridization signal of microdissected DNA probes in the C-positive region is determined by the high concentration of repetitive sequences and the incomplete hybridization suppression of the repetitive sequences present in the DNA probes. The CISS hybridization signal of the 5^RC2 DNA probe in the C-negative region of chromosome 5^R adjacent to the heterochromatic block and in the short arm of chromosome 5 is caused by the presence of the material from the euchromatic region of the chromosome in the DNA probe that got into it during microdissection upon generation of the 5^RC2 DNA probe. The signals in the distal region of the short arm of chromosome 5 and in the euchromatic region adjacent to the C-positive heterochromatic block of chromosome 5^R, obtained as a result of CISS hybridization of the 5^RC2 DNA probe, suggest the presence of homologous unique DNA sequences in their composition, i.e., the preservation of the position of the chromosome 5 distal region during the formation of chromosome 5^R. These data are not consistent with the hypothesis on the change in the position of the centromeric region as a result of the chromosome 5 short arm inversion.

Comparative Analysis of the Organization of the Short Arm of Chromosome 5 and Proximal Euchromatic Region of the Long Arm of Chromosome 5^R

To determine the order of homologous regions in the short arm of chromosome 5 and proximal euchromatic region of the long arm of chromosome 5^R, a pair of overlapping microdissected DNA probes (DNA probes 5-1, 5-2) was obtained from the short arm of chromosome 5 and the proximal region of its long arm (the scheme of chromosome dissection is shown in Fig. 1). Chromosome fragments were dissected so that different number of collected copies corresponded to different parts of the dissection region, with the maximum number of collected copies corresponding to the selected chromosome region within the dissection region (in Fig. 1, indicated by a thickened line). The number of collected copies decreased in the direction
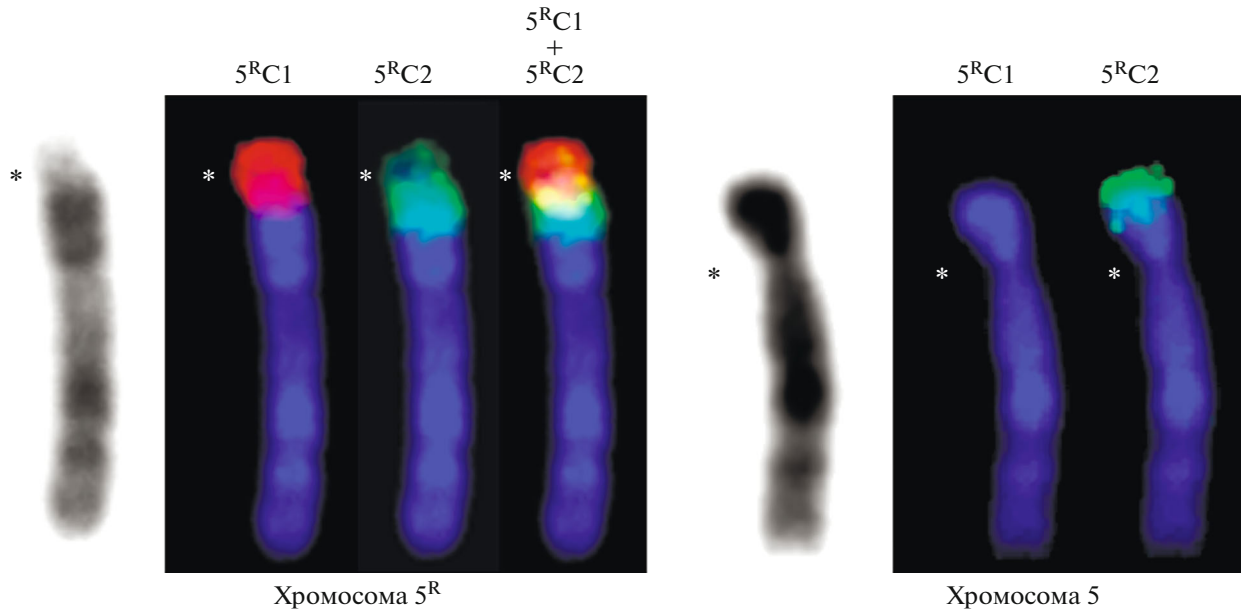
Fig. 2. FISH with the 5^RC1 and 5^RC2 DNA probes on chromosomes 5 (right) and 5^R (left) of *M. arvalis* obscurus. The inverted DAPI banding patterns are presented. The DNA probe signals combined with DAPI staining are shown (5^RC1, red signal; 5^RC2, green signal). The centromeric regions are designated by asterisks.

of the dissection region boundaries. In CISS hybridization, DNA probes obtained in this way gave maximum signal intensity in the region with maximum number of collected copies with a gradual decrease in the signal intensity to the dissection region boundaries. In multicolor FISH experiments, this provided a unique ratio of signal intensities of the used DNA probes in different segments of the studied region of chromosome 5.

Two-color FISH on *M. arvalis* obscurus metaphase chromosomes using DNA probes 5-1 and 5-2 provided painting of the pter → p13 and p15 → q13 regions of chromosome 5 and their homologous regions of chromosome 5^R (Fig. 3). The ratio of relative signal intensities of these DNA probes characterized individual segments within painted regions. Segments containing homologous unique sequences were visualized using a software program (MSV, the ISIS3 software package from METASYSTEMS GmbH). Generation of pseudocolored chromosome segments was based on the ratios of the DNA probe relative signal intensities, i.e., on the composition of unique DNA sequences in the analyzed regions [14].

The pseudocolor nomenclature was generated in such a way as to ensure reliable identification of chromosome segments. The sizes of these segments appeared to be considerably smaller than the region detected by the microdissection-derived DNA probe, which made it possible to compare the order of the identified homologous segments in chromosomes 5 and 5^R. The comparison showed that, during the chromosome 5 reorganization, there was the centromeric

transposition without the inversion of regions in its short arm (Fig. 3).

It is also noteworthy that the appearance of an additional C-block in chromosome 5^R probably took place without amplification of the pericentromeric repeats of chromosome 5, since microdissected DNA probe 5-2, which included DNA from this region, gave no signal in the additional C-positive region. The lack of the 5^RC1 signal in the distal region of the short arm of chromosome 5 suggests that, initially, there was no noticeable cluster of repeats, the amplification of which could lead to the formation of part of a large additional heterochromatic block located in the short arm of chromosome 5^R. This suggestion is consistent with the absence of a signal during the hybridization of DNA probes, including pericentromeric (DNA probe 5-2) and peritelemeric (DNA probe 5-1) regions of chromosome 5, in the additional C-positive heterochromatic region of chromosome 5^R (Fig. 3).

Clusters of Repetitive Sequences Homologous to the Repeats of the Additional C-Positive Heterochromatic Block of M. arvalis obscurus Chromosome 5^R in Chromosomes of Voles of the Subgenus Microtus

The results of FISH with the 5^RC1 and 5^RC2 DNA probes (Fig. 2) suggest that these probes contain the repeats as follows: telomere-associated repeats (DNA probe 5^RC1); ribosomal genes; repeated sequences characteristic of pericentromeric repeats; other repeats, including dispersed repeats, characteristic of euchromatin regions of chromosomes (DNA probe 5^RC2).
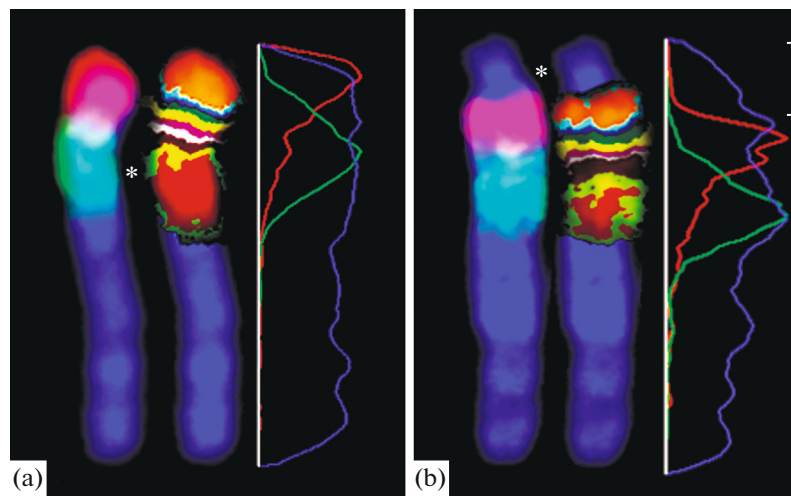
Fig. 3. CISS hybridization of the DNA probes (red signal) and 5-2 (green signal) and multicolor banding of the regions of chromosomes 5 (a) and 5^R (b) of *M. arvalis* obscurus. To the right of the chromosomes, relative intensity profiles of the hybridization signals (red and green lines) and DAPI staining (blue line) are shown. Additional C-positive heterochromatic block of chromosome 5^R is indicated by a bracket; the centromeric regions are designated by asterisks.

The differences in the localization of FISH signals of the 5^RC1 and 5^RC2 DNA probes in the additional C-positive region of chromosome 5^R should be mentioned. The 5^RC1 DNA probe painted the whole C-positive region of chromosome 5^R, including NOR, while the 5^RC2 DNA probe gave an intense signal in the part of the C-positive heterochromatic block located in the long arm of chromosome 5^R, only slightly painting the material of its short arm. FISH with the 5^RC1 and 5^RC2 DNA probes was performed on metaphase chromosomes of both chromosomal forms of *M. arvalis*, as well as on chromosomes of *M. rossiaemeridionalis*, *M. kirgisorum*, and *M. transcaspicus* (Fig. 4).

The 5^RC1 DNA probe produced no specific signals either on chromosome 5 of *M. arvalis* or on its homologs in other studied vole species (Fig. 4). The identification of chromosomes homeologous to chromosome 5 of *M. arvalis* was carried out according to the results of FISH with the 5^RC2 DNA probe, which labeled homeologous euchromatin regions of chromosomes in all studied species.

The dissection region for the generation of the 5^RC1 DNA probe contained active NOR, which suggested the presence of the DNA fragments homologous to the ribosomal genes in the DNA probe. However, FISH with the 5^RC1 DNA probe gave signals not in all active NORs previously identified in the chromosomes of *M. arvalis* [9], and in some of them, only weak specific signals were detected (Figs. 4a, 4b). This is probably associated with the fact that ribosomal DNA constituted only a small part of the dissected C-positive region. Thus, FISH with the 5^RC1 DNA probe allows for tracing the distribution of repeat clusters homologous to heterochromatin of *de novo*

formed heterochromatic region in chromosomes of voles, but does not provide reliable identification of all clusters of ribosomal genes. To designate the repeat clusters detected by FISH with the 5^RC1 and 5^RC2 DNA probes, hereinafter, the abbreviations "C1 repeat clusters" and "C2 repeat clusters" were used.

The results of FISH with the 5^RC1 and 5^RC2 DNA probes showed the difference in the number, size, and localization of the C1 and C2 repeat clusters in different chromosomal forms of *M. arvalis*. Surprisingly, the chromosomal form *M. arvalis* arvalis was characterized by a higher number and larger sizes of these clusters, while a large additional block of heterochromatin containing these repeats was detected in the reconstructed chromosome 5^R of *M. arvalis* obscurus. Large clusters of C1 repeats were identified in pericentromeric regions of small- and medium-sized acrocentric chromosomes of this species (Figs. 4a, 4b). In *M. arvalis* arvalis, large clusters of C2 repeats were present on the same chromosomes. They were localized distal to the C1 repeat clusters (Fig. 4a). In addition, in *M. arvalis* arvalis, clusters of C2 repeats were detected in pericentromeric regions of several small biarmed chromosomes (Fig. 4a). In the chromosomes of *M. arvalis* obscurus, these clusters were detected only in acrocentric chromosomes near clusters of C1 repeats, and their sizes were considerably smaller (Fig. 4a). In the chromosomes of other studied vole species, FISH with the 5^RC2 DNA probe did not reveal clusters of repeated sequences. The only exception was chromosome 1 of *M. rossiaemeridionalis*. This chromosome is a homeolog of chromosome 5 of *M. arvalis*, which differs from the latter with the short arm inversion, which led to the transposition of centromere to the telomeric region [9]. In some copies of *M. rossiaemeridionalis* chromosome 1, FISH with the 5^RC2 DNA probe gave
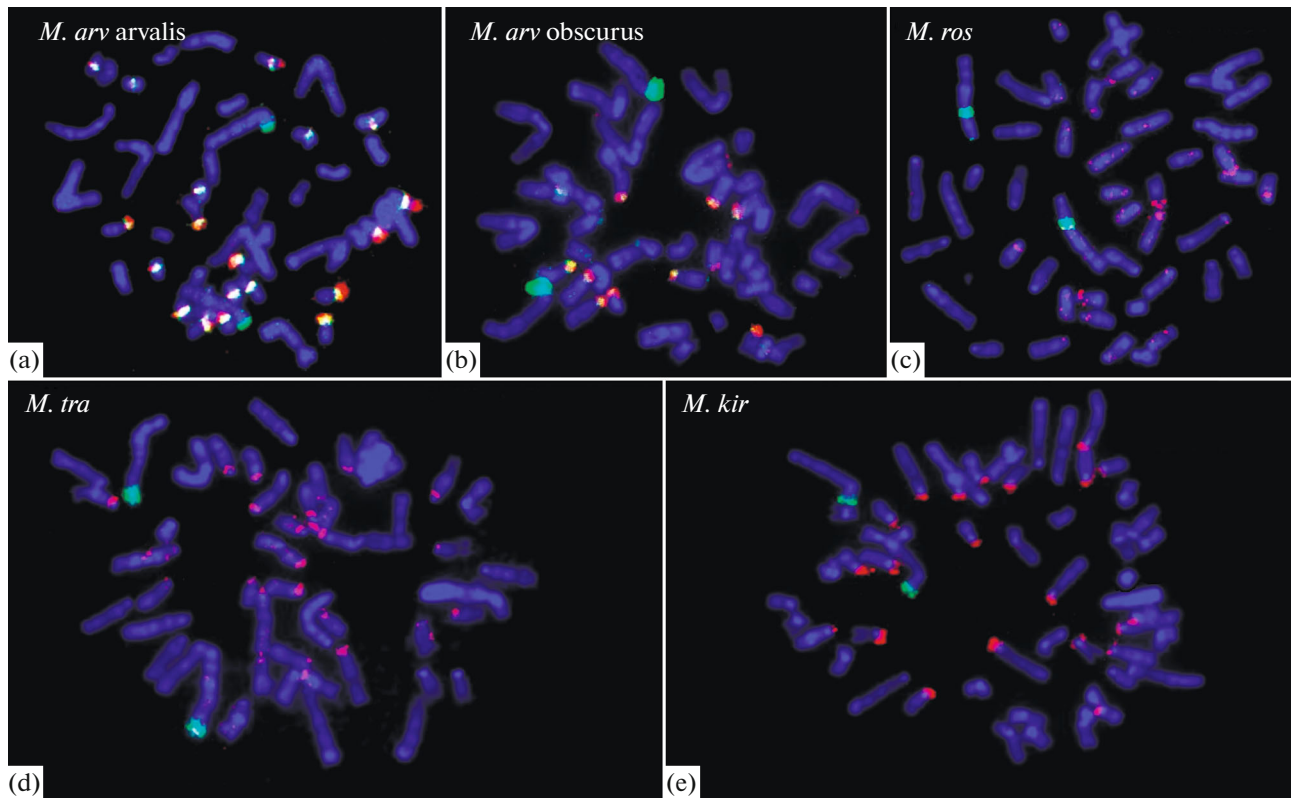
Fig. 4. Two-color FISH with the 5^{R}C1 (red signal) and 5^{R}C2 (green signal) DNA probes on metaphase chromosomes of *M. arvalis* arvalis (a), *M. arvalis* obscurus (b), *M. rossiaemeridionalis* (c), *M. transcaspicus* (d), and *M. kirgisorum* (e).

a small but specific signal in the pericentromeric region indicating the presence of a small cluster of C2 repeats in this region (Fig. 4).

Clusters of repeats homologous to C1 repeats were identified in pericentromeric regions of a considerable number of acrocentric chromosomes of *M. rossiaemeridionalis*, *M. kirgisorum*, and *M. transcaspicus*. The highest number of chromosomes with such clusters was found in *M. kirgisorum*. In addition, their localization in *M. kirgisorum* differed from that in chromosomes of *M. rossiaemeridionalis* and *M. transcaspicus*. In *M. kirgisorum*, clusters of repeats homologous to C1 are localized in the short arms of a part of the acrocentric chromosomes (Fig. 4e), while in *M. rossiaemeridionalis* and *M. transcaspicus*, these clusters, also being present in acrocentric chromosomes, are localized in the long arms, in the distal parts of pericentromeric C-positive regions (Figs. 4c, 4d).

Clusters of Repetitive Sequences Homologous to the Repeats of the Additional C-Positive Heterochromatic Block of M. arvalis obscurus Chromosome 8^R, in the Chromosomes of Voles of the Subgenus Microtus

In the additional C-positive region of chromosome 8^R, FISH with the 5^{R}C1 DNA probe gave weak scattered signal while the signal of the 5^{R}C2 DNA probe did not exceed the background level (Fig. 5). On the basis of microdissection of two regions of chromosome 8^R and DNA amplification of isolated regions, two DNA probes were obtained with DOP-PCR, including 8p (microdissection of the short arm) and 8^{R}C (microdissection of the C-positive region) (Fig. 1). CISS hybridization with the 8p DNA probe intensely painted short arms of chromosome 8 and 8^R. FISH with the 8^{R}C DNA probe painted the whole additional C-positive region (Fig. 5). The 8p DNA probe was later used to paint regions in chromosomes 8 and 8^R in *M. arvalis* and in homeologous chromosomes in other vole species (Fig. 6).

FISH with the 8^{R}C DNA probe intensely painted the distal region of the q-arm of chromosome 8 of *M. arvalis* obscurus and its homeologous regions in chromosomal of other studied vole species (Fig. 6). In the additional C-positive heterochromatic block of chromosome 5^R of *M. arvalis* obscurus, the FISH with 8^{R}C DNA probe painted only the nucleolar organizer region (Fig. 7). It can be suggested that the additional C-positive region of chromosome 8^R contained a large number of sequences homologous to rDNA and they appeared to be present in the 8^{R}C DNA probe. This resulted in signals in many NORs on the chromo-
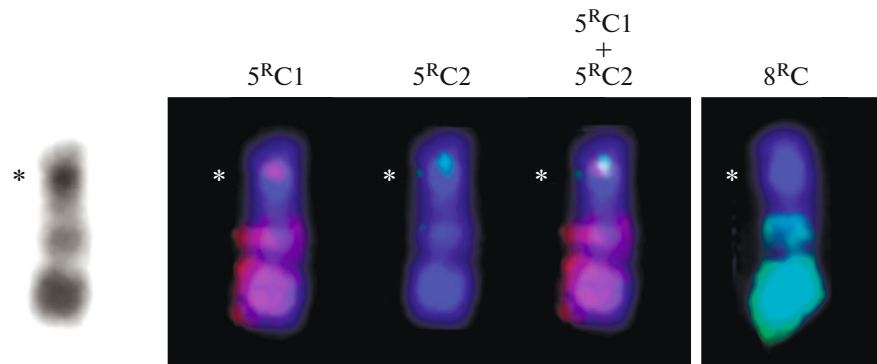
Fig. 5. FISH with the 5^RC1, 5^RC2, and 8^RC DNA probes on chromosome 8^R of *M. arvalis* obscurus. From left to right: inverted DAPI banding, the 5^RC1 (red signal) and 5^RC2 (green signal) DNA probes, and combined signals of DNA probes and FISH 8^RC DNA probe. The centromeric regions are designated by asterisks.
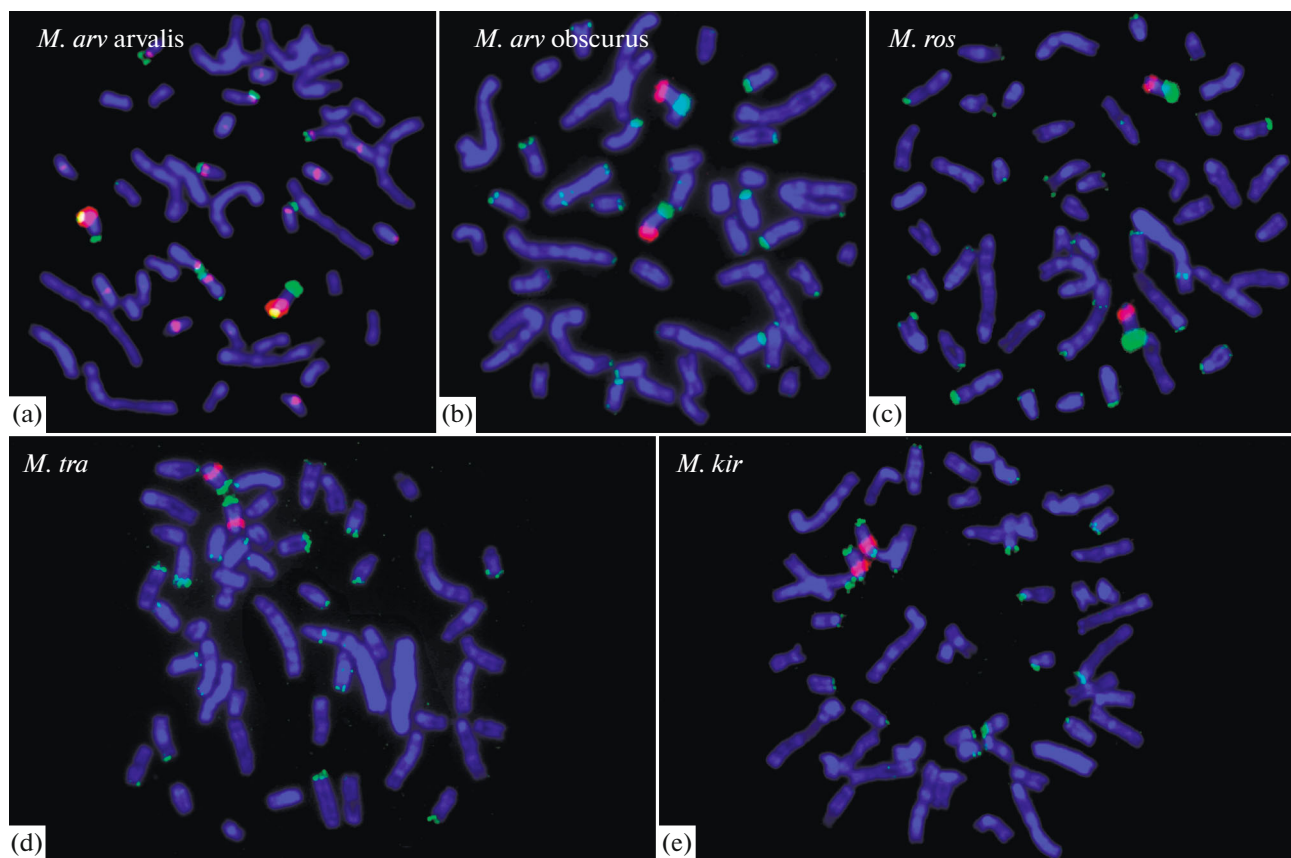

Fig. 6. Two-color FISH with the 8p DNA probes (red signal) and the 8^RC DNA probe (green signal) on metaphase chromosomes of *M. arvalis* arvalis (a), *M. arvalis* obscurus (b), *M. rossiaemeridionalis* (c), *M. transcaspicus* (d), and *M. kirgisorum* (e).

somes of all studied vole species (Fig. 6) after FISH with the 8^RC DNA probe. However, it should be noted that the smallest NORs avoided detection upon performing FISH with the 8^RC DNA probe. It seems likely that the hybridization signal of the 8^RC DNA probe was largely determined by the ribosomal genes and repetitive sequences associated with the vole NORs. In chromosomes of voles, FISH with the 8^RC DNA probe revealed also clusters of telomere associated repeats, which could be localized on both long and short arms of the chromosomes. In addition to a large cluster in the distal region of the long arm of chromosome 8 of *M. arvalis* obscurus and its homeologous regions in other vole species, the 8^RC DNA

Fig. 7. FISH with the 8^RC DNA probe (green signal) on metaphase chromosome 5^R of *M. arvalis* obscurus. The centromeric region is indicated by an asterisk.

probe painted teleromeric regions of 11 pairs of autosomes and X chromosome in *M. arvalis* obscurus, three pairs of autosomes and X chromosome in *M. arvalis* arvalis, 12 pairs of acrocentric chromosomes in *M. rossiaemeridionalis*, and 16 pairs of autosomes in *M. transcaspicus*. After FISH with the 8^RC DNA probe, in teleromeric chromosome regions of *M. kirgisorum*, no signals were detected, with the exception of NORs (Fig. 6). Moreover, in the chromosomes of any of the studied vole species, FISH with this DNA probe gave no signals in pericentromeric heterochromatin regions.

CISS hybridization with the 8^RC DNA probe on metaphase chromosomes of voles of the *arvalis* group produced minor signals in the interstitial chromosome segments. FISH with the 8^RC DNA probe did not paint C-positive heterochromatic regions of sex chromosomes (Fig. 6).

The DNA probe derived from 8p included the material of pericentromeric heterochromatin region of this chromosome. FISH with this DNA probe on the chromosomes of *M. arvalis* arvalis, in addition to painting the short arm of chromosome 8 and pericentromeric heterochromatin in the long arm, produced intense signals in the pericentromeric heterochromatin regions of several acrocentric and biarmed chromosomes. Surprisingly, such signals were not detected in any chromosome of *M. arvalis* obscurus. In the chromosomes of the other studied vole species, CISS hybridization with this DNA probe also painted only euchromatic homeologous chromosome regions.

DISCUSSION

Analysis of repeat distribution in the chromosomes of common voles suggests that, during the evolution in this group of species, insertions, transpositions, and amplification of different repeated DNA sequences took place. The elucidation of the integration time of a small number of copies of alien sequences into the genomes of the studied species is beyond the scope of this study. We consider that visualization of such sequences by the FISH technique becomes possible only after they form clusters, or the chromosomal regions become considerably enriched in them. There are no doubts that additional C-positive heterochromatic blocks in chromosomes 5^R and 8^R of *M. arvalis* obscurus contained not one but several types of repeated sequences. This is indicated by FISH with the 5^RC1 and 5^RC2 DNA probes, which reveals different regions of chromosomes enriched in repeats. Obviously, the additional C-positive region of chromosome 8^RC also contains different repeated sequences.

It seems likely that the formation of clusters of repetitive elements homologous to the DNA found in additional C-positive heterochromatic blocks of *M. arvalis* obscurus in the chromosomes of voles of the group *arvalis* began relatively recently, already after the separation of the species group *agrestis* of the subgenus *Microtus*. This is indicated by the complete absence of signals after FISH with the 5^RC1 and 5^RC2 DNA probes on metaphase chromosomes of *M. agrestis* (species from the group *agrestis* of the subgenus *Microtus*) [4].

The unexpected result was the identification of an extended and intense signal in the distal part of chromosome 8 in both chromosomal forms of *M. arvalis* and in homeologous chromosome regions of other studied vole species upon performing FISH with the 8^RC DNA probe and in the absence of detectable C-heterochromatic blocks in these regions. Identification of such signal can be explained in terms of the presence in this region of a cluster of corresponding repeated sequences already in ancestral species with the subsequent dispersal of these repeats to the telomeric regions of other chromosomes. We suppose that these regions may contain rather extended duplicons, including unique vole genome sequences. This hypothesis can be tested by conducting FISH with the 8^RC DNA probe on chromosomes of more distant vole species, in the genomes of which there was no amplification and dispersal of the repeats that constitute the main part of the additional C-positive region from which the 8^RC DNA probe was generated.

The results obtained so far provide only qualitative size estimates of the corresponding C-positive blocks and clusters of repeated sequences, as well as their diversity estimates in pericentromeric and telomeric chromosome regions. Detailed analysis of their distri-

bution and determination of the copy number requires cloning of the DNA fragments that are the microdissected DNA library members, generation of the DNA probes on their basis, and the performance of FISH. Sequencing of cloned DNA fragments and FISH with the obtained DNA probes on metaphase chromosomes of different vole species will make it possible to proceed to the analysis of specific regions of the vole genome at the level of sequenced repeats.

The results from this study point to differences in the formation of clusters of repetitive sequences in closely related species and even in different chromosomal forms of the same species. FISH with appropriate microdissected DNA probes made it possible to analyze the localization of such repeat clusters and to show that they differed in both the time of dispersal and the regions of preferred localization. The dispersal of repeats present in one of these clusters was observed only in *M. arvalis*, while the others were represented in numerous chromosome regions of all studied vole species (Figs. 4, 6). Clusters of repeats homologous to repeats of the additional C-positive region in the long arm of chromosome 5^R were present in pericentromeric regions of some chromosomes of this species, but were not found in the other studied vole species. In *M. arvalis*, repeats characteristic of the additional C-positive heterochromatic region, localized in the short arm of chromosome 5^R, were found mainly in the short arms of acrocentric chromosomes. However, it should be noted that localization of homologous repeat clusters in different species was different. In *M. arvalis* and *M. kirgisorum*, they were localized only in the short arms of acrocentric chromosomes; in *M. rossiaemeridionalis*, they were localized preferably in the distal part of C-positive pericentromeric regions of the long arms; and in *M. transcaspicus*, these clusters were localized almost equally as often in the chromosome short and long arms. Unfortunately, it is impossible to determine the dispersal patterns of these repeats in the chromosomes of the ancestors of the species studied. It can be suggested that, in different ancestors, the regions of preferred formation of these clusters were different, but it cannot be excluded that the localization of repeat clusters was changed later owing to subsequent chromosomal rearrangements. Nevertheless, analysis of the chromosomal distribution of repeat clusters that are homologous to the DNA of the short arm of chromosome 5^R makes it possible to get back to the issue of NOR localization in the chromosomes of voles of the *arvalis* group. Previously, it was demonstrated that, in acrocentric chromosomes of *M. arvalis* and *M. kirgisorum*, NORs were localized in the short arms, whereas in the homeologous acrocentric chromosomes of *M. transcaspicus* and *M. rossiaemeridionalis*, they were localized in the pericentric regions of the long arms. This almost coincides with the localization of clusters of repetitive sequences involved in this study. Considering the patterns of cluster distribution revealed by the FISH with

5^RC1, 8^RC probes and Ag-NOR staining and also taking into account uniform intraspecific NOR location, it can be suggested that in chromosomes of one pair of species we observed an ancestral variant of NOR location, whereas in the other pair of species, transposition of rDNA took place. Moreover, such transposition took place in all NOR-bearing chromosomes. It is difficult to imagine that transposition could have resulted from concerted inversions in the pericentromeric regions of these chromosomes. It is more likely that the transposition of repeats, including ribosomal genes, into new regions and their subsequent amplification occurred in the ancestors of a pair of species that changed their position relative to the centromere. Moreover, their amplification resulted in an excessive number of ribosomal genes, which provoked the subsequent loss of part of the ribosomal genes in both new and old NORs. Since the repeat amplification occurred only in new regions, such dynamics of the ribosomal gene number resulted in the complete loss of old NORs. The participation of repeats present in the short arm of chromosome 5^R in the formation of new NORs is indicated both by the presence of active NOR in the heterochromatic block itself and the total increase in the number of NORs in *M. arvalis obscurus* compared to *M. arvalis arvalis*.

Comparative analysis of the autosome GTG banding patterns in voles of the *arvalis* group [9] showed that, during the evolution, Robertsonian fusions and a small number of inversions of chromosomal regions took place. With the exception of one inversion in chromosome 15, in *M. kirgisorum*, inversion of the whole chromosome arms took place; i.e., inversions were formed with the involvement of chromosome regions including clusters of repeats analyzed in the present study. It seems likely that enrichment of chromosome regions in these repeats led to an increase in the probability of chromosomal rearrangements. This suggestion agrees well with the enrichment in these repeats of the chromosomal rearrangement hotspots identified in the X chromosomes of voles of the *arvalis* group [4].

Reviewing the analysis of the distribution patterns of repeat clusters in the studied vole species, it should be noted that repetitive DNA in pericentromeric and telomeric regions of chromosomes of these species are one of the most rapidly evolving part of the genome, as was shown in the analysis of other mammalian species [1, 2, 7]. Numerous studies of the mechanisms of formation of the interphase nucleus general architectonics, necessary for normal cell functioning [15–17], and meiosis [18, 19] have shown the important role of pericentromeric and telomeric regions in their realization. Thus, rapid DNA divergence in these regions, even upon relatively short-term geographic isolation of the populations, can lead to fundamental divergence in the genome organization and preservation of reproductive isolation during reunification of the ranges of

temporarily isolated populations. The process of speciation is extremely complex and, probably, there are different pathways of the appearance of reproductive isolation. The proposed mechanism is only one of the many possible scenarios that can lead to speciation. However, it is interesting in that it explains the existence of great differences that are observed in different taxa, and indicates the need to analyze repetitive sequences during the study of the mechanisms and directions of chromosomal evolution in different mammalian taxa and, especially, in low-ranking taxa.

FUNDING

COMPLIANCE WITH ETHICAL STANDARDS

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflicts of interest. The authors declare that they have no conflicts of interest.

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Translated by N. Maleeva