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Variation in highly repetitive DNA composition in rye and wild relatives discovered by FISH

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Aim. Determination of the sequence organization of the chromosomes terminal regions in *Secale cereale* L. varieties and some of its wild relatives for further identification of individual chromosomes with the use of appropriate probes. **Methods.** Fluorescence *in situ* hybridization, microscopy. **Results.** FISH analysis revealed 26–28 sites of the pSc200 sequence at the ends of all 14 pairs of chromosomes and 18 signals of the pSc250 sequence on the chromosomes of *S. cereale* in four accessions. Repeats were differently localized on the chromosomes of the closely related species *Dasypyrum villosum* and *Dasypyrum breviaristatum*, so it can be assumed that the tetraploid *D. breviaristatum* has an allopolyploid origin, may not be a descendant of *D. villosum.* **Conclusions.** A characteristic distribution of pSc200 and pSc250 tandem repeats of the tribe *Triticeae* was established, which allows revealing the evolutionary relationships between the studied species and the directions of their divergence.

K e y w o r d s: *Secale cereale* L., *Dasypyrum* species*, Agropyron cristatum* L., subtelomeres, tandem repeats, fluorescence *in situ* hybridization.

Introduction

One of the ways to expand the genetic diversity of wheat, rye or barley is the transfer of genes of economically valuable traits from closely related genera and species, united into three genetic pools: primary (wheat varieties), secondary (various types of *Triticum* and *Aegilops*), tertiary (the most distant species of *Triticeae*) [1].

Wild relatives can serve as donors not only of the genes responsible for resistance to stress factors, but also can raise the yield by increasing fertility, the number of ears and other indicators, as well as improve the quality of final products due to new variants of storage proteins. Molecular and molecular-cytogenetic markers

allow the transfer of target genes or chromatin regions, as well as tracking their introgression into the genome in segregated populations. Thus, recombination in interspecific chromosome associations could be promoted with the aim of transferring desirable agronomic traits from related genetic donor species into crops [2]. Additionally, recombination operates at subtelomeres and, as it happens in rye and wheat, homologous recognition and pairing are more often correlated with the recombining regions than with the crossover-poor regions [3].

Repetitive DNA is the most variable part of the genome, which causes significant differences between genomes and reflects evolutionary distances between species [4, 5]. A classification of the tandem repeats was created, dividing them into two classes: dispersed and tandemly repeated sequences. The latter are usually localized in centromeres and telomeres. The number of such DNA repeats corresponds to the size of heterochromatin regions of chromosomes. Tandem repetitive DNA exhibits wide diversity in sequence, even between closely related species, and is considered as a part of the rapidly evolving eukaryotic genome [6]. Knowledge of nucleotide composition and genomic organization, chromosomal location and evolutionary origin of repetitive DNA sequences is necessary for understanding the organization, behaviour and functional significance of sequences in eukaryotic genomes, for evolution and establishment of phylogenetic relationships between species [7], identification and taxonomy of plants [8], as well as to control the purity of varieties [9].

Large heterochromatin blocks in subtelomeric regions are a characteristic chromosomal feature of rye (*Secale cereale* L.), which its closest relatives (wheat and barley) of the *Triticeae* tribe do not have. At the end of the last century, it was discovered that some tandemly organized families have a large number of monomer copies, as indicated by strong *in situ* hybridization signals. Together, these families make up to 8–12 % of the entire genome of *S. cereale* [10]. The molecular structure, copy number, and length of the monomers of the three most common repeats — pSc119.2, pSc200, and pSc250, which consist of monomer units with a length of 118, 379, and 571 bp, respectively, were also determined [11]. The results of fluorescence *in situ* hybridization (FISH) revealed pSc200 and pSc250 close to telomeres, whereas some copies of pSc119.2 are confined to interstitial regions. The pSc119.2 sequence is also present in some other cereals, but pSc200 and pSc250 are largely specific for *S. cereale*.

While intervarietal heterogeneity in the distribution of highly repetitive DNA in rye is well documented, the comparison between different hybrids and varieties is still of interest. Therefore, we compare the rye chromosome polymorphism in four varieties and in the two genera *Dasypyrum* and *Agropyron* to study the sequence organization of subtelomeric regions in these species for further identification of individual chromosomes with the use of appropriate probes.

Materials and Methods

Plant material. The starting materials for the study were the seeds of *S. cereale* varieties Petkus and Imperial from John Innes Centre (Norwich, UK), the rye cultivars Selgo from Czech Republic and a self-pollinated stock of the Ukrainian rye Zhyttedaine, as well as the seeds of *Dasypyrum villosum* L., *Dasypyrum breviaristatum* (Lindb) Frederiksen and *Agropyron cristatum* L., obtained from the staff

of the Hryshko National Botanical Garden of the National Academy of Sciences of Ukraine.

For molecular genetic analysis, the preparations were made from the apical meristem of the roots, 0.8–1 cm long. In order to accumulate and synchronize mitoses, the roots were kept at a temperature of +4 °C for 24–28 h. The samples were fixed in a mixture of ethanol:glacial acetic acid in a ratio of 3:1. The pressed preparations were checked using phase-contrast microscopy.

DNA probes and their labeling. Two non-homologous subtelomeric sequences pSc200 and pSc250 were used to study the spatial molecular organization of telomere-associated heterochromatin of *S. cereale*. Both probes were labelled by polymerase chain reaction (PCR) using the forward and reverse M13 primers. Amplification products were checked on an agarose gel and cloned into the pCRII-TOPO vector (Invitrogen).

Fluorescence in situ hybridization. The chromosomal preparations and *in situ* hybridization were performed according to the method [12]. On each slide with metaphase chromosomes, 20 ml of the hybridization mixture with a denatured labelled probe were applied and covered with a coverslip. Denaturation was performed at a temperature of 80 °C for 2 min. Further, hybridization was carried out in a hermetically closed humid chamber at 37 °C for 16 h. After that, the coverslip was washed, the preparations were kept in 2xSSC buffer for 10–15 min, washed and air-dried. The biotin-labeled samples were detected using streptavidin, and the digoxigenin-labeled samples were detected using anti-digoxigenin fluorescein [13]. The preparations were counterstained with the fluorescent dye DAPI (4,6-diamidine-2-phenylindole) and analyzed on a Leica fluorescence microscope with appropriate filters using the ISIS program (Metasystems, Altlussheim, Germany).

Results and Discussion

Staining of chromosomes using DAPI made it possible to detect heterochromatin regions of chromosomes in all studied species. The obtained results show that the pSc200 tandem repeat is localized on the terminal regions of two arms of all seven pairs of *S. cereale* chromosomes (2n = 14), and the pSc250 repeat is located on the terminal regions of the short arms of all chromosomes and on the long arms of chromosomes 1R and 3R in the most rye varieties. FISH analysis revealed 28 signals of the pSc200 sequence at the ends of all 14 pairs of chromosomes and 18 signals of the pSc250 sequence (Fig. 1a–c). Differences were found in Imperial and Zhyttedaine accessions (Tab. 1).

Heterochromatin regions in *D. villosum* (2n = 14) are located mainly at the terminal regions, as well as near the centromere of chromosomes. Intercalary sites of heterochromatin are present on some chromosomes (Fig. 1d–f). In *D. breviaristatum* (2n = 28) it was found that only individual chromosomes have the sites of clear constitutive heterochromatin at the end. Its demonstration was observed in centromeric regions and gradient staining of individual chromosomes along the entire length (Fig. 1h).

The location of the pSc200 sequence in *D. villosum* has distinct tandem nature of organization on chromosomes, being localized mainly on the subtelomeric regions (Fig. 1f). The pSc250 repeat has a dispersed location: many signals of this repeat are detected on some chromosomes and almost absent on others (Fig. 1e). Therefore, it is possible to identify chromosomes in the *D. villosum* karyotype by DAPI staining and hybridization with pSc200.

Both pSc200 and pSc250 sequences have different patterns of organization on *D. brevia-*

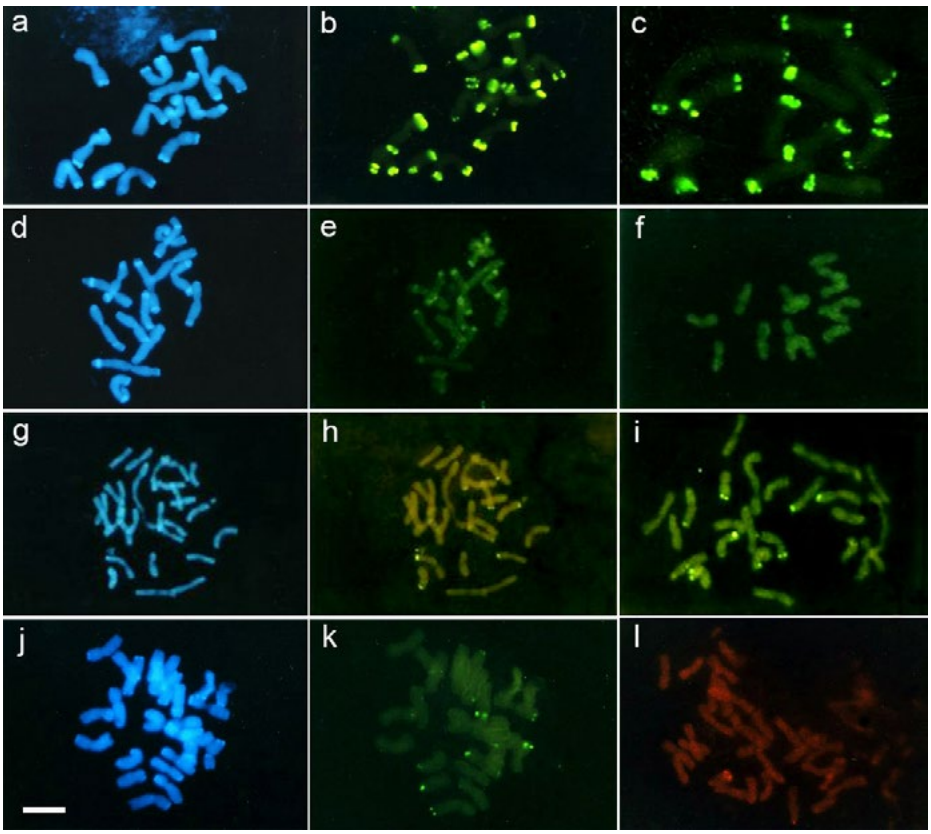

Fig. 1. Metaphase plates of apical meristem cells of *Secale cereale* (a–c), *Dasypyrum villosum* (d–f), *Dasypyrum breviaristatum* (g–i) and *Agropyron cristatum* (j–l). Slides with chromosomes were stained with DAPI (blue) — a, d, g, j, hybridized with pSc200 (yellow-green fluorescence) — b, e, h, k and pSc250 (yellow-green fluorescence & red fluorescence) — c, f, i, l.

Table 1. **The distribution of pSc200 and pSc250 DNA repeats on the chromosomes of four rye varieties**

Chromosomes		**pSc200**			**pSc250**		
Pair	Arm	Petkus/Selgo	Imperial	Zhyttedaine	Petkus/Selgo	Imperial	Zhyttedaine
1	Short	+	+	+	+	+	+
	Long	+	+	+	+	+	+
2	Short	+	+	+	+	+	+
	Long	+	+	+	–	–	–
3	Short	+	+	+	+	+	+
	Long	+	+	+	+	+	+
4	Short	+	+	+	+	+	+
	Long	+	–	+	–	–	–
5	Short	+	+	+	+	+	+
	Long	+	+	–	–	–	–
6	Short	+	+	+	+	+	+
	Long	+	+	+	–	–	–
7	Short	+	+	+	+	+	+
	Long	+	+	+	–	+	–

Comment: + presence; – absence of repeats signal

ristatum chromosomes (Fig. 1g–i). 12 pSc250 sites and 10 pSc200 sites on separate chromosomes were detected. From the obtained data it is clear that in *D. breviaristatum* it is possible to distinguish the chromosomes that have localization sites of pSc200 and pSc250 sequences and those that do not have them.

It is known from the literature that the sequences of pSc200 and pSc250 have a tandem nature of location and joint colocalization on separate chromosomes [14]. Based on the fact that the pSc200 and pSc250 repeats are located differently on the chromosomes of closely related *Dasypyrum* species — *D. villosum* and *D. breviaristatum*, it can be assumed that the tetraploid *D. breviaristatum* has an allopolyploid origin. As a result of FISH analysis with repeated sequences of pSc200 and pSc250, the authors showed reverse hybridization patterns with two probes [14]. Thus, there is now evidence to demonstrate that diploid *D. villosum* is not the progenitor of *D. breviaristatum*.

The nature of the distribution of constitutive heterochromatin in *A. cristatum* (2n = 28) resembles the distribution in *D. breviaristatum* with distinct sites near the telomeres of some chromosomes. In *A. cristatum*, the pSc200 and pSc250 repeats have different localization on chromosomes (Fig. 1j–l). The pSc200 marked about 10 sites near telomeres and numerous sites in intercalary heterochromatin. The pSc250 sequence has a dispersed pattern of arrangement. An equally high number of signals are observed on all chromosomes of *A. cristatum*.

It is known that in *S. cereale*, the location of subtelomeric pSc200 and pSc250 sequences is specific for each of the arms of chromosomes with respect to the order of domains and the distribution of spacers [13]. In wild cereals, such as *D. villosum*, *D. breviaristatum* and *A. cristatum*, the nature of the distribution of heterochromatin regions is different from that of *S. cereale*. For a long time, the question of whether these species have repetitive sequences of the subtelomeric heterochromatin of *S. cereale*, and how they are located on the chromosomes, remained controversial. There is now no doubt that the most common subtelomeric heterochromatin sequences of *S. cereale* are located on the chromosomes of these wild species [15].

It was shown that the pSc200 and pSc250 repeats present in the genomes of some *Dasypyrum* species differ in the number of copies [16]. *D. villosum* has a copy number of pSc200 close to that of *S. cereale*, the content of pSc200 and pSc250 in *D. breviaristatum* and *A. cristatum* as well as pSc250 in *D. villosum* was significantly lower, detected by blot hybridization and *in situ* hybridization. Although being close relatives, wheat and barley still have few copies of these sequences, which can only be detected by PCR, their localization is not visualized by Southern blot hybridization or FISH analysis [16].

All these data indicate that evolutionary changes in subtelomeric heterochromatin of pSc200 and pSc250 sequences occur at different levels. These chains of sequences were amplified or shortened to varying degrees in genomes during speciation. Dover suggested that a random process of genomic rearrangements leads to the selective amplification of certain DNA sequences [17]. Accordingly, natural selection favours the formation of tandemly repeated regions that likely confer some advantage in genome stabilization to gene expression, meiotic fusion, and recombination. However, it remains unclear why the same sequence is amplified in one genome and scarce in another.

There is an assumption that where the telomeric repeats are in large blocks, the chromatin takes the form of heterochromatin. The correlation between the number of copies of pSc200 and pSc250, the intensity of the *in situ* hybridization signals and the size of the heterochromatin blocks are consistent with the chromatin resulting from the high concentration and homogenization of tandem DNA sequences with the primary structure occurring in the evolution of *S. cereale* [18].

With the help of FISH analysis, high variations in signal intensity were found not only between the studied species, but also among different chromosomes within the same species [14, 19]. Amplified DNA usually contains the structures such as novel junctions and inverted repeats. The peculiarity of these structures is that they, like the heterogeneous monomers pSc200 and pSc250 in wild *Triticeae* species, can trigger the secondary amplification mechanisms. A sharp contrast in the accumulation of pSc200 and pSc250 in some species of the tribe *Triticeae* belonging to the same genus indicates that each family of repeats is an independent unit of evolution, and perhaps different mechanisms are involved in the amplification of such sequences [18, 20]. The chromosomal domains consisting of pSc200 and pSc250 sequences are usually separated by non-tandem DNA sequences. Restriction DNA analysis of BAC clones in a BAC library of the short arm of chromosome 1R of *S. cereale* followed by blot hybridization showed that the pSc200 and pSc250 sequences, like human alpha-satellite DNA, form higher order repeats (HORs), each consisting of 2–8 monomers. One HOR is longer than 3 kb (379 bp×8) in pSc200 and is almost 3.5 kb (571 bp×6) in pSc250. The order of HORs and the ratio of different HORs are specific within one arm [10, 21]. This means that multiple recombinations occurred independently in different regions of the same arm of the chromosome, which led to the appearance of the HOR.

It is likely that the variations in the number of copies of these classes of repeats are associated with the formation of specific sections of chromosomes, which are important for their behaviour and stabilization of the species-specific karyotype [9]. Thus, the evolution of these regions can occur independently of the regions that contain many genes. The characteristic location of tandem repeats in the terminal regions of plant chromosomes indicates genetic affinity and provides a basis for the selection of a certain variety or line [22]. Emden noted that subtelomeres are highly polymorphic and therefore less conserved than chromosome ends. Also, the genes are very abundant, and recombination is more frequent in these regions [23]. FISH experiments showed that, in the absence of subtelomeric sequences, the chromatin remodeling failed and homologous chromosomes would not recognize and pair [24]. The relevant function of subtelomeres in recombination was also shown in the experiments using wheat lines with distal chromosome deletions [25].

New organizations of subtelomeric tandem repeats on 1RS were found, and they reflected new genetic variations of 1RS arms [3]. These 1RS arms might contain abundant allelic diversity for agricultural traits. The narrow genetic base of 1RS arms in 1BL.1RS and 1AL.1RS translocations currently used in agriculture is seriously restricting their use in wheat breeding programs. This research has found new 1RS sources for the future restructuring the 1BL.1RS translocations [2]. The allelic variations of these

1RS arms should be studied more intensely as they may enrich the genetic diversity of 1BL.1RS translocations. And the study of this region allows us to see some changes, for example, a decrease in the size of 1R in Imperial and Zhyttedaine [9], which led to the possibility of flow sorting of this chromosome and the creation of the 1R and 1RS library [21]. Additionally, there are the reports in the literature that chromosome 5R of *S. cereale* contains some elite genes, which can be used to improve wheat cultivars [26]. Namely, the long arm of chromosome 5R contains the genes for resistance to stripe rust, which may be novel, and their location differs from those previously reported.

Conclusions

Thus, our research showed a different nature of the distribution of heterochromatin regions in the chromosomes of wild cereals (*D. villosum*, *D. breviaristatum* and *A. cristatum*) compared to *S. cereale*. The characteristic distribution of the tandem repeat sequences of pSc200 and pSc250 of the studied varieties was established. The study of the Zhyttedaine variety showed a unique possibility for sorting the 1R chromosome separately from the rest, and the isolation of this line led to the deletion of the pSc200 repeat on the long arm of the 5R chromosome. The obtained results are necessary for further identification of individual chromosomes, establishment of relationships between the studied species and the direction of divergence within the *Triticeae* tribe.

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Author Contributions

O.G.A. conceived and wrote the paper; M.O.T. and P.A.P prepared material, methodology, analysis; V.A.K. funding acquisition and resources. All authors have read and agreed to the published version of the manuscript.

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Варіації високоповторюваних послідовностей ДНК, виявлених методом FISH, у жита та його диких родичів

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Мета. Визначення послідовності організації термінальних ділянок хромосом чотирьох сортів *Secale cereale* L. та деяких його диких родичів для подальшої ідентифікації окремих хромосом з використанням відповідних зондів. **Методи.** Флуоресцентна гібридизація *in situ*, мікроскопія. **Результати.** За допомогою FISH аналізу виявлено 26–28 сайтів послідовності pSc200 на кінцях усіх 14 пар хромосом та 18 сигналів послідовності pSc250 на хромосомах *S. cereale*. Ці повтори були по-різному локалізовані на хромосомах близькоспоріднених видів *Dasypyrum villosum* та *Dasypyrum breviaristatum*, тому припускаємо, що тетраплоїд *D. breviaristatum* має аллополіплоїдне походження і не може бути нащадком *D. villosum*. **Висновки.** Виявлено характерний розподіл тандемних повторів pSc200 та pSc250 триби *Triticeae*, що дозволяє встановити еволюційні зв'язки між досліджуваними видами та напрямки їх дивергенції.

Ключові слова: *Secale cereale* L., види *Dasypyrum, Agropyron cristatum* L., субтеломери, тандемні повтори, флуоресцентна гібридизація *in situ*.

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