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Liberties of the genome: insertions of mitochondrial DNA fragments into nuclear genome

M.V. Golubenko, V.P. Puzyrev

Research Institute of Medical Genetics, Tomsk National Research Medical Center of the Russian Academy of Sciences, Tomsk, Russia
maria-golubenko@medgenetics.ru

Abstract. The transition of detached fragments of mitochondrial DNA into the nucleus and their integration into chromosomal DNA is a special kind of genetic variability that highlights the relation between the two genomes and their interaction in a eukaryotic cell. The human genome contains several hundreds of insertions of mtDNA fragments (NUMTS). This paper presents an overview of the current state of research in this area. To date, evidence has been obtained that the occurrence of new mtDNA insertions in the nuclear genome is a seldom but not exceptionally rare event. The integration of new mtDNA fragments into the nuclear genome occurs during double-strand DNA break repair through the non-homologous end joining mechanism. Along with evolutionarily stable "genetic fossils" that were integrated into the nuclear genome millions of years ago and are shared by many species, there are NUMTS that could be species-specific, polymorphic in a species, or "private". Partial copies of mitochondrial DNA in the human nuclear genome can interfere with mtDNA during experimental studies of the mitochondrial genome, such as genotyping, heteroplasmy assessment, mtDNA methylation analysis, and mtDNA copy number estimation. In some cases, the insertion of multiple copies of the complete mitochondrial genome sequence may mimic paternal inheritance of mtDNA. The functional significance of NUMTS is poorly understood. For instance, they may be a source of variability for expression and splicing modulation. The role of NUMTS as a cause of hereditary diseases is negligible, since only a few cases of diseases caused by NUMTS have been described so far. In addition, NUMTS can serve as markers for evolutionary genetic studies. Of particular interest is the meaning of NUMTS in eukaryotic genome evolution. The constant flow of functionally inactive DNA sequences from mitochondria into the nucleus and its significance could be studied in view of the modern concepts of evolutionary theory suggesting non-adaptive complexity and the key role of stochastic processes in the formation of genomic structure.

Key words: mitochondrial DNA; nuclear copies of mtDNA; NUMTS; genome evolution; mtDNA inheritance.

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Вольности генома: инсерции фрагментов митохондриальной ДНК в ядерный геном

М.В. Голубенко, В.П. Пузырёв

Научно-исследовательский институт медицинской генетики, Томский национальный исследовательский медицинский центр Российской академии наук, Томск, Россия
maria-golubenko@medgenetics.ru

Аннотация. Переход отдельных фрагментов митохондриальной ДНК в ядро и встраивание их в ДНК хромосом являются особым типом генетической изменчивости, характеризующим связь и взаимодействие двух геномов эукариотической клетки. В геноме человека содержится несколько сотен таких инсерций (NUMTS). Статья посвящена обзору современного состояния исследований в этой области. К настоящему времени получены данные о том, что появление новых инсерций мтДНК в ядерном геноме – редкое, но не исключительное событие. Встраивание новых фрагментов мтДНК в ядерный геном происходит при репарации двунитевых разрывов ДНК по механизму негомологичного соединения концов. Наряду с эволюционно стабильными «генетическими ископаемыми», встроившимися в ядерный геном миллионы лет назад и общими для многих видов и более крупных таксонов, существуют видоспецифичные, полиморфные и «приватные» NUMTS. Копии фрагментов митохондриальной ДНК в ядерном геноме человека могут интерферировать с митохондриальной ДНК при экспериментальных исследованиях митохондриального генома, таких как генотипирование и изучение ге-

тероплазмии отдельных вариантов мтДНК, анализ метилирования мтДНК, определение числа копий мтДНК в клетке. Кроме того, в некоторых случаях инсерция нескольких копий полной последовательности митохондриального генома может имитировать наследование мтДНК от отца к детям. Вопрос о функциональной значимости NUMTS остается малоизученным. В частности, они могут являться источником изменчивости для модуляции экспрессии и сплайсинга. Роль NUMTS как причины развития моногенной наследственной патологии невелика, поскольку описано всего несколько случаев заболеваний, обусловленных NUMTS. Помимо этого, NUMTS могут служить маркерами для эволюционно-генетических исследований. Отдельный интерес представляет значение NUMTS в эволюции генома эукариот. Постоянный поток функционально неактивных последовательностей ДНК из митохондрий в ядро и его значение можно исследовать с точки зрения современных представлений теории эволюции, связанных с неадаптивностью сложности и центральной ролью стохастических процессов в формировании структуры геномов.

Ключевые слова: митохондриальная ДНК; ядерные копии мтДНК; NUMTS; эволюция генома, наследование мтДНК.

Introduction

Mitochondrial DNA (mtDNA), which is situated outside the cell nucleus, is a special part of the genome. The establishment of symbiosis between the ancestor of the eukaryotic cell and the ancestor of the mitochondrion was the most important event in biological evolution, leading to the emergence of eukaryotes. During the further evolution of eukaryotes, most genes moved from mitochondria to the nucleus. This process apparently began immediately after the introduction of alphaproteobacteria into the cytoplasm of pro-eukaryotes (see review (Panov et al., 2020)). Moreover, it is assumed that the mosaic structure of eukaryotic genes is a consequence of the integration of DNA fragments from endosymbionts into the nuclear genome at the early stages of eukaryotic evolution, which, in turn, stimulated cell compartmentalization and isolation of the nucleus (Koonin, 2006; Rogozin et al., 2012).

Genomes of modern mitochondria contain a very limited set of genes. In most animals, mtDNA encodes only 13 subunits of respiratory chain proteins, ribosomal and transfer RNAs. The remaining genes have long and irreversibly "moved" into the nucleus. However, comparative genomic analysis shows that the integration of new mtDNA fragments into the nuclear genome continues, now being a microevolution process. So, in the chromosomal DNA of modern eukaryotes, including humans, there are many regions that are homologous to the mitochondrial genes. These sequences are called NUMTS – NUclear MiTochondrial Sequences. The placement of NUMTS in the genome is often associated with repetitive elements and transposons, but NUMTS themselves are not mobile genetic elements. The "mission" of NUMTS has not yet been revealed, but they are of interest both in a practical sense, because they may have a pathogenic effect, and in a theoretical aspect, since they may represent a different path of genome evolution.

The article is devoted to an overview of the current state of research on the NUMTS phenomenon and its role in the life of the human genome.

Prevalence of NUMTS in the human genome

Soon after the sequence of human mitochondrial DNA was determined, DNA fragments embedded in nuclear chromosomes and homologous to mtDNA were discovered (Tsuzuki et al., 1983). As the human genome was sequenced, the analysis of homology between NUMTS and modern mtDNA in humans and other species showed that the insertion of mtDNA fragments into chromosomes is an ongoing process (Mourier et al., 2001). NUMTS are found on all human chromosomes and are situated mostly in regions rich in various repeats. Development of new sequencing technologies, improvements in bioinformatics, and the accumulation of data on individual genomes lead to the identification of more and more such insertions, and it is becoming evident that NUMTS is a common phenomenon. The human reference genomic sequence GRCh37/hg19 contains 766 insertions of mitochondrial genome fragments homologous to the modern human mtDNA reference sequence (Calabrese et al., 2012). Subsequently, analysis of data from the 1,000 Genomes Project (999 individuals from 20 populations) identified 141 polymorphic NUMTS sites in the nuclear genome, in addition to those insertions that are "fixed" in the human population. Of these, 42 % of polymorphic NUMTS were located in introns, 43 % were located in intergenic regions, and most of these NUMTS were "younger" than a million years old (Dayama et al., 2014).

A recent analysis of the complete genomes of 66,000 individuals, including more than 10,000 trios (Wei et al., 2022), has already identified more than 1,500 new NUMTS, the vast majority of which were rare in the population or "private", i. e. found in only one individual. So, the incidence of *de novo* NUMTS insertions has been estimated to be approximately 1 in 10,000 births and approximately 1 in 1,000 tumors. Moreover, estimates of the time of integration into the nuclear genome obtained for several hundred NUMTS showed that 90 % of these events occurred no more than 100 thousand years ago (Wei et al., 2022). Some figures characterizing the diversity of NUMTS in the human genome are presented in Table 1. It is worth noting that the total length of NUMTS is about 630,000 bp (Tao et al., 2023), or approximately 0.02 % of the total length of the human genome.

Depending on the search and aligning algorithms, the minimal length of detected mtDNA fragments starts from 30 bp, and most of them are shorter than 500 bp. However, insertions of almost the entire mitochondrial genome sequence also occur. In particular, in the intergenic region on chromosome 4, there is an insertion 14,836 bp in length, homologous to a 14,904 bp region in the mtDNA sequence (positions 661–15,564) (Calabrese et al., 2012).

Mitochondria are not the only organelles that "send" fragments of their DNA to the nucleus. To the same extent, this

Table 1. Characteristics of the NUMTS "landscape" in the human nuclear genome

Study	Number of NUMTS	Median length (bp)	Cumulative length (bp)	Average homology with mtDNA, %
Ramos et al., 2011	755	225	548,250	79.2
Calabrese et al., 2012	766	214	541,113	79.5
Wei et al., 2022	1,637 (including polymorphic)	156	ND	ND
Tao et al., 2023	863	194	631,156	ND
Uvizl et al., 2024	846	ND	548,500	80.9

Note. ND – no data.

process is characteristic of plastids (Zhang et al., 2024). In addition, this phenomenon may be more or less prevalent depending on the species. For example, the search for NUMTS in the genomes of 13 different species revealed large interspecific differences: the nematode, some dipterans (*Anopheles*, *Drosophila*) and puffer fish have only a few mtDNA fragments in their nuclear genome, while humans, some insects, and plants have several hundred NUMTS (Richly, Leister, 2004; Leister, 2005). Moreover, the number of NUMTS may depend on the genome size and speciation characteristics.

These data suggest that the integration of mtDNA fragments into chromosomal DNA is not a rare event but a natural property of the human genome dynamics, and therefore it must be taken into account and should be explored.

The mechanism for the emergence of new NUMTS

Almost all studies show that the general mechanism for the integration of mtDNA fragments into the nuclear genome is non-homologous end joining (NHEJ) as a way to repair double-stranded DNA breaks (Gaziev, Shaikhaev, 2010). Usually, NUMTS are associated with mobile genetic elements: for example, a study of 271 human NUMTS showed that most of them are located within 150 bp from repetitive elements (predominantly LINE and Alu repeats) or even within these sequences (Mishmar et al., 2004). A recent search and analysis of NUMTS in the genomes of 45 mammalian species has essentially confirmed this fact (Uvizl et al., 2024).

In a study from Japan (Onozawa et al., 2015), it was shown that DNA insertions belonging to the second class of "templated sequence insertion polymorphism" (TSIP) had some characteristics consistent with their occurrence as a result of double-strand breaks repair event with use of the mechanism of non-homologous end joining, and it is noteworthy that in more than 20 % of TSIP cases, mitochondrial DNA served as the "donor" DNA for such insertions (Onozawa et al., 2015).

According to the results of experiments on irradiation of chicken eggs, new insertions of mtDNA fragments were identified in 25 % of surviving embryos (2 out of 8) (Abdullaev et al., 2013). In the paper on the case of a pathogenic *de novo* NUMTS insertion leading to the development of Pallister–Hall syndrome, the authors note that the family where the affected child was born lived in an area exposed to the Chernobyl accident in 1986 (Turner et al., 2003). It is fair to assume that since ionizing radiation leads to double-strand breaks in DNA and the appearance of new NUMTS is associated with the process of repairing this damage, the probability of integration of mtDNA fragments into the nuclear genome increases after irradiation.

It should be noted that in a non-dividing cell, the nuclear and mitochondrial genomes are separated from each other by a total of four membranes (the nuclear double membrane and the mitochondrial double membrane). To integrate a fragment of mtDNA into the DNA of a chromosome, this fragment should be able to enter the nucleus. To date, several possible ways of such transfer have been proposed. The most acceptable hypothesis is the assumption that mtDNA fragments that appear due to the impact of reactive oxygen species enter the cytoplasm as a result of changes in the mitochondrial membrane (opening of pores, mitochondrial fusion/fission, etc.), and then are transported into the nucleus using vacuoles (Puertas, González-Sánchez, 2020).

NUMTS studies in evolutionary genetics

Depending on the time of their origin, NUMTS can provide information about the evolutionary history of the human species (Hazkani-Kovo, 2009). Two features of the evolution of NUMTS can be distinguished in comparison with the homologous mtDNA regions: firstly, NUMTS are pseudogenes, therefore selection does not affect them, and the mutation process is more "uniform", and secondly, the rate of molecular evolution declines after integration into the nuclear genome, consistent with general differences in mutation rates between nuclear and mitochondrial DNA. That is, on the one hand, the "biological clock" for NUMTS works more precisely, and on the other hand, they are a kind of "genetic fossil" containing information about mtDNA haplotypes that might not have been preserved in modern populations, so they can serve as an "outgroup" for intraspecific phylogeny (Bravi et al., 2006). For example, two NUMTS in the human genome that are homologous to the *CO1* gene contain nucleotide substitutions (compared to the reference mtDNA sequence) characteristic of the most ancient mitochondrial superhaplogroup L (Mishmar et al., 2004).

Using a comparative analysis of polymorphic NUMTS in the genomes of *Homo sapiens sapiens*, *H. sapiens neanderthalensis* and *H. sapiens denisova*, five insertions of mtDNA

fragments were identified. These insertions occurred during the evolution of the genus *Homo* and have been preserved in the genomes of modern humans. Of these, two NUMTS originated from the mitochondrial genome of Denisovans and entered the modern human genome as a part of nuclear DNA. They were identified in the genomes of several Indonesians (Bücking et al., 2019). Analysis of NUMTS in the genomes of great apes revealed several fragments, for which the divergence of their sequence from modern mtDNA of these species indicated that they also entered the genomes of hominids as part of nuclear DNA due to admixture of unknown extinct species (Popadin et al., 2022). Interestingly, an analysis of the time of appearance of *Homo*-specific NUMTS in the human genome showed that the occurrence of a significant number (one third of the 18 analyzed) of insertions coincided in time with the estimated time interval of the origin of the genus *Homo*, as well as with drastic climate change, i. e. about 2.5–2.9 million years ago. Thus, speciation appears to be associated with an increase in the rate of insertion of new NUMTS into the genome. However, the question remains open whether these insertions are just markers of a period of genomic instability in the species' history ("riders") or whether they play a significant role in speciation, changing the structural and expression architecture of the genome ("drivers") (Gunbin et al., 2017). The first hypothesis is supported by data on a similar "explosion" in the frequency of NUMTS in marsupial martens, which occurred during the same period (Hazkani-Covo, 2022). The second hypothesis deserves attention due to the fact that NUMTs are often found in regions of open chromatin associated with DNase I hypersensitivity and expression regulation (Wang, Timmis, 2013). The uneven rate of organelle DNA insertions into chromosomes during evolution is also demonstrated by homology analysis of NUMTS and the "parental" organelle genomes: the distribution of NUMTS by extent of their sequence identity to the mitochondrial genome shows that although these insertion events occur throughout the species history, the rate of the process is not constant. For example, in *Homo sapiens*, most NUMTS have 70 to 85 % identity with the mitochondrial genome, while in *Phytophthora*, the sequence identity is about 100 % (Hazkani-Covo, Martin, 2017).

Pathogenic effects of NUMTS

Random insertion of any DNA fragment into exonic or regulatory regions of genes can have a pathogenic impact. Cases of hereditary diseases caused by *de novo* insertions of mtDNA fragments into nuclear genes have indeed been described, but it should be noted that they are rare (Table 2).

The first case of a disease associated with NUMTS was described in 2002. Severe coagulation factor VII deficiency was found in a patient who was a compound heterozygote: one copy of the gene had a 7 bp deletion, and the other had a 251 bp insertion from the *MT-RNR1* gene into the polypyrimidine tract near the splice acceptor site in intron 4 of *F7* (Borensztajn et al., 2002). In 2003, a sporadic case of Pallister–Hall syndrome was characterized: a *de novo* 72-bp insertion from mtDNA into exon 14 of the *GLI3* gene resulted in a frameshift and the formation of a premature stop codon (Turner et al., 2003). Notably, the allele with this *de novo* NUMTS was of paternal origin. In addition, several other cases of pathogenic NUMTS disrupting splice sites or causing frameshifts have been published. Given the large number of genetic tests being performed (targeted and exome sequencing) that can potentially detect such insertions, we can conclude that pathogenic NUMTS in humans are extremely rare.

In contrast to the few cases of NUMTS leading to hereditary diseases and syndromes, *de novo* insertions within exons and regulatory sequences in malignant tumors are not so rare. In one study, 220 somatic "tumor-specific" NUMTS were identified within genes, and out of these, 13 were located in the coding regions of genes (including 3 and 4 that disrupted stop and start codons, respectively), and 16 were located in

Table 2. Known cases of diseases caused by insertions of mtDNA fragments

Disease	Gene	Event	Reference
Factor VII deficiency	*F7*	Insertion of a 251 bp fragment from *MT-TF* and *MT-RNR1* genes (591–809) which occurred near acceptor splicing site in intron 4, resulting in exon 5 skipping	Borensztajn et al., 2002
Usher syndrome IC	*USHIC*	A 36 bp insertion into exon 9, originating from the *MT-TL2* gene (12,253–12,288)	Ahmed et al., 2002
Pallister–Hall syndrome	*GLI3*	*De novo* 72 bp insertion into exon 14, originating from *MT-TS2* and *MT-TL2* (12,244–12,315), causing a frameshift and a premature stop codon	Turner et al., 2003
Mucolipidosis type IV	*MCOLN1*	Insertion of 93 bp from *MT-ND5* into exon 2, resulting in splicing disruption	Goldin et al., 2004
Lissencephalia	*PAFAH1B1*	Insertion of 130 bp from the sequence of *MT-ATP8* (8,479–8,545) and *MT-ATP6* (8,775–8,835) into exon 2 just upstream of the translation initiation site	Millar et al., 2010
The X-linked hyper-IgM syndrome	*CD40LG*	Insertion of 147 bp from *MT-RNR1* (664-805) into exon 1, causing a translation frameshift and a premature stop codon	Li X. et al., 2021

the 3′ or in 5′ untranslated regions (Wei et al., 2022). Possibly, accumulation of somatic NUMTS with time may also contribute to aging.

Recently, it was shown that insertions of mtDNA fragments into introns can affect gene expression, i. e. transcription and splicing, especially if the inserted fragments contain tRNA genes that are capable of forming secondary structures. In particular, one study examined the effect of such mitochondrial tRNA (nimtRNA) gene insertions on splicing, using a splicing reporter gene construct (Hoser et al., 2020). The experiments showed that nimtRNAs inserted into the intron of the reporter gene enhance pre-mRNA splicing, depending on their number and location, as well as the efficiency of splice site recognition, while the insertion of nuclear tRNAs did not have such an effect. In addition, this work demonstrated that partial deletion of nimtRNA(Lys), located in intron 28 of the *PPFIBP1* gene, reduces the likelihood of inclusion of exon 29 in the mRNA (Hoser et al., 2020). Thus, some NUMTS may have a regulatory impact.

NUMTS as a source of artifacts in mitochondrial DNA studies

MtDNA heteroplasmy

When studying mtDNA heteroplasmy, NUMTS can significantly interfere with the results, especially in the case of low levels of the mutant allele (Maude et al., 2019; Xue et al., 2023). In particular, G. Dayama et al. (2014) identified 59 positions in mtDNA where false heteroplasmy caused by polymorphic insertions in the nuclear genome can be systematically detected. A comparison of enrichment methods for NGS (hybridization or long-range PCR) and alignment approaches (aligning reads on the whole genome or only on mtDNA, using different threshold levels for heteroplasmy detection) showed that a significant part of the "alternative" alleles in heteroplasmic positions actually correspond to NUMTS alleles, and this effect is more pronounced when using a low heteroplasmy threshold, a hybridization enrichment method, and mtDNA as the only reference for alignment. On the other hand, taking these factors into account leads to a decrease in coverage depth and to the omission of truly heteroplasmic positions in mtDNA (Li M. et al., 2012).

For the sample of a thousand individuals from the Swedish population, analysis of complete mitochondrial genomes showed that with an average mtDNA read depth of more than 2000x, about 40 % (373 out of 934) of mtDNA haplotypes have "heteroplasmic" positions with a variant fraction of more than 2 % (i. e. above the "noise level") that is driven by variants in NUMTS (Sturk-Andreaggi et al., 2023). At the same time, 31 "heteroplasmic" positions were characterized by a proportion of the alternative (associated with NUMTS) allele of more than 10 %, but the authors note that in these cases the mtDNA reading depth was less than 100x (Sturk-Andreaggi et al., 2023). Given that mtDNA mutations leading to the development of mitochondrial diseases are also heteroplasmic, and the level of heteroplasmy can vary depending on the tissue, it is important to take NUMTS existence into account when performing genetic diagnostics (Yao et al., 2008).

NUMTS and assessment of mitochondrial DNA methylation level

Studies of the mitochondrial DNA epigenetics produce contradictory results: some groups of researchers reveal a fairly high level of cytosine methylation in mtDNA, while others reveal a very low methylation level (see reviews (Byun et al., 2013; Hong et al., 2013; Zinovkina and Zinovkin, 2015; Maresca et al., 2015; Patil et al., 2019)). Analysis of these publications suggests that the resulting estimates of the proportion of methylated cytosines depend on the detection methods. Since NUMTS are essentially pseudogenes, they are expected to be methylated, and this is indeed supported by direct determination of methylation levels using Oxford NanoPore technology (Wei et al., 2022). In particular, our own studies showed an extremely low (at the scale of error rate) level of cytosine methylation in the regulatory region (D-loop) of mtDNA; this estimate was obtained by sequencing (NGS) of PCR products using sodium bisulfite-treated DNA as a template (Golubenko et al., 2018).

Recent publications have shown that the true level of cytosine methylation in mtDNA is indeed less than 1 %, and higher values are caused by "interference" of signals from nuclear pseudogenes (i. e. NUMTS) or the influence of nucleotide context on base calling (Bicci et al., 2021; Guitton et al., 2022; Shao et al., 2023). However, it should be noted that DNA methyltransferase DNMT1 was in fact found in mitochondria, and its mitochondrial isoform is synthesized from an alternative promoter (Shock et al., 2011). So, the existence of DNA methylation in mitochondria cannot be completely excluded, and for instance, it might occur during programmed or pathological deactivation/degradation of mtDNA.

NUMTS and mtDNA copy number estimation

The presence of NUMTS is a major difficulty in the development and use of methods for quantifying mtDNA copy number per cell, i. e. the ratio of the number of copies of a mtDNA region to the number of copies of a "control" nuclear gene. Currently, several methods are used to determine mtDNA copy number, the most popular of them is real-time PCR using fluorescent dyes, including TaqMan probes, as well as digital PCR. Designing primers that could anneal only to mtDNA involves significant difficulties, since almost the entire mtDNA sequence is represented in the nuclear genome, and moreover, a significant part of it is represented by a large number of fragments, sometimes comparable to the number of mtDNA copies in the cell. In addition, each individual lacks on average four NUMTS from the reference genome sequence (Wei et al., 2022). Thus, even a thorough BLAST analysis for the primers and probes sequences that takes into account sequence identity level of NUMTS and mtDNA, adding the high level of polymorphism of mtDNA itself, does not always allow to make an adequate assessment of the mtDNA copy number in a cell. Probably, several regions of mtDNA should be used simultaneously for these purposes.

NUMTS and forensic studies

Considering that events of *de novo* insertion of mtDNA fragments into the nuclear genome are not extremely rare and

their length can be large, data obtained by forensic experts during molecular genetic examinations should be interpreted with caution. If a large mtDNA insertion persists in the genome for several generations, or if a child "inherits" part of the parent's mtDNA genotype in its nuclear genome due to a *de novo* insertion, then analysis of a total DNA sample will yield a mixture of the two haplotypes (see, for example, Lutz-Bonengel et al., 2021) and could potentially lead to false DNA identification. In addition, co-amplification of NUMTS can probably occur in other cases (for example, when analyzing a degraded DNA sample, where the copy number of mtDNA is low and comparable to the copy number of homologous NUMTS in the sample under study (Bravi et al., 2006)). It was shown that when analyzing data obtained using multiple parallel sequencing (NGS, or MPS) methods, it is possible to filter out NUMTS using bioinformatics methods, but in forensic studies, researchers often deal with degraded DNA samples from which only short fragments can be obtained, and in this case bioinformatic "filtering" is less effective (Marshall, Parson, 2021).

"Paternal inheritance" of mtDNA

The history of the search for the possibility of paternal inheritance of human mtDNA is quite interesting. Reports on cases with a supposed contribution of mtDNA from sperm mitochondria to the general pool of mtDNA in the zygote and developing organism keep appearing in the scientific press. A recent sensational publication on this topic (Luo et al., 2018) demonstrated three pedigrees where children inherited their father's mtDNA in a certain proportion and then passed it to some of their children in the same proportion. The authors suggested that the possibility of paternal mtDNA inheritance was due to a variant of a nuclear gene with a dominant effect. This paper gave rise to an extensive scientific debate in the following publications (Luo et al., 2019; Lutz-Bonengel, Parson, 2019; McWilliams, Suomalainen, 2019), and also stimulated further research in this area, which showed that these cases can be explained by insertions of concatemers (tandem linear copies) of mtDNA into the nuclear genome, representing the so-called mega-NUMTS (Wei et al., 2020; Bai et al., 2021). For example, one concatemer identified on chromosome 14 consisted of 50 copies of mtDNA (Lutz-Bonengel et al., 2021).

And yet, the final verdict on the topic of "intergenerational transmission of the paternal mitochondrial genome" should not be rendered, since it is still unclear how exactly the obligate elimination of paternal mtDNA is ensured in the zygote. Some studies show that there is no universal mechanism for such elimination. For example, in nematodes, sperm mitochondria are "digested" in the zygote after fertilization using the autophagy mechanism, but if it does not happen, then the embryos are not viable. In mice (and, probably, humans), the paternal mitochondrial genome is eliminated already in the mitochondria of the sperm, which, therefore, do not contain mtDNA at all. However, if for some reason mtDNA is not completely degraded, then its presence in the mouse embryo can be traced up to the morula stage (Luo et al., 2013).

The results of experiments on the introduction of human mtDNA into mouse zygotes using microinjections, conducted at the Institute of Experimental Medicine in St. Petersburg, attract attention in this regard. In some embryos and newborn mice, human mtDNA was retained in some tissues, and in some cases, it was transmitted to F1 and even F2 offspring (Sokolova et al., 2004; Bass et al., 2006). Later it was demonstrated that mouse and human mitochondria successfully merged with each other in cell fusion experiments, and also produced "xenocybrids" containing the mouse cell nucleus and human mitochondria, although they could not grow in a medium requiring normal mitochondrial function (Yoon et al., 2007). Thus, the formation of chimeric human and mouse mitochondria is possible, and it is likely that after microinjection into the mouse zygote, human mitochondria were combined with mouse ones. It is unknown whether human mtDNA was integrated into mice nuclear genome in this case, and additional experiments are needed to clarify this, but given that human mtDNA was found in much less than half of the offspring and not in all tissues, and that injections of mitochondria were carried out into already fertilized zygotes, it can be assumed that it was not contained in the nucleus but specifically in the mitochondria.

Conclusion

The phenomenon of translocation of mtDNA fragments into the nuclear genome is a special type of genomic variability that deserves close attention. In recent years, it has been shown that the prevalence of these events is much higher than previously thought. The mitochondrial genome unexpectedly appeared not as a subordinate "prisoner" of the eukaryotic cell, but as an independent source of new material for the nuclear genome. The role of this phenomenon in the life of the cell remains unknown. Perhaps its understanding goes beyond the framework of classical "deterministic" genetics and can be explored in the paradigm of a new "postmodern" approach, which assumes the multiplicity of patterns and processes of evolution for the living forms, as well as the central role of unpredictable events, that is, the non-adaptiveness of the main path of evolution (Koonin, 2014). This suggests the need for stochastic transformation of the genome in evolution, "genomic instability" (Khesin, 1985) or "genome liberties" (Puzyrev, 2002). It is worth noting that while genetics as a science was developing in the frame of classical simplified concepts regarding genes, mutations and heredity, the ideas of gene mobility, as well as abruptness of mutational changes and multiplicity of gene manifestations at the phenotypic level, were expressed by many researchers since the end of the 19th century (see in: Puzyrev, 2002; Golubovsky, 2011).

It is interesting that in the model of the evolution of entropy and genome complexity proposed by E.V. Koonin, two scenarios are considered, including a "high-entropy" way, which is accompanied by a decrease in gene density, and the opposite "low-entropy" way, which consists of genome optimization and maximum information density (Koonin, 2014). We can say that the transfer of mtDNA fragments into the nuclear genome contributes to its evolution in the "high-entropy" mode, while the mitochondrial genome itself followed the opposite "low-entropy" scenario. It is noteworthy that these two paths are governed, among other things, by the effective population size, which is small in the first case ("high-entropy") and

large in the second ("low-entropy"); this rule surprisingly corresponds to the diploidy (in most cases) of the eukaryotic nuclear genome, on the one hand, and the large number of mitochondria inhabiting them, on the other hand. It should also be noted that it is the simplification of the genome following an abrupt increase in its complexity that is assumed by this model as a general trend in evolution (Wolf, Koonin, 2013), and "an increase in the entropy of the genome ... can be considered as a "genomic syndrome", as the inability of organisms with a small effective population size to cope with the spreading of selfish elements and other processes leading to the increase in entropy" (Koonin, 2014).

If we consider NUMTS from a "practical" point of view, it has now been demonstrated that nuclear copies of mitochondrial DNA fragments in the human genome can introduce some noise into the data obtained from experimental studies of the mitochondrial genome, but may also carry some functional load. At least, they serve as a variability source for modulation of expression and splicing. In addition, they have significant potential as polymorphic markers for evolutionary genetic studies. Also, NUMTS may be involved in speciation, but this issue requires further research. The significance of NUMTS in the development of monogenic hereditary pathology is apparently small, and their role in aging and the development of multifactorial diseases, including cancer, remains to be studied.

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A hypothesis about interrelations of epigenetic factors and transposable elements in memory formation

R.N. Mustafin

Bashkir State Medical University, Ufa, Russia
ruji79@mail.ru

Abstract. The review describes the hypothesis that the drivers of epigenetic regulation in memory formation are transposable elements that influence the expression of specific genes in the brain. The hypothesis is confirmed by research into transposon activation in neuronal stem cells during neuronal differentiation. These changes occur in the hippocampus dentate gyrus, where a pronounced activity of transposons and their insertion near neuron-specific genes have been detected. In experiments on changing the activity of histone acetyltransferase and inhibition of DNA methyltransferase and reverse transcriptase, the involvement of epigenetic factors and retroelements in the mechanisms of memory formation has been shown. Also, a number of studies on different animals have revealed the preservation of long-term memory without the participation of synaptic plasticity. The data obtained suggest that transposons, which are genome sensors highly sensitive to various environmental and internal influences, form memory at the nuclear coding level. Therefore, long-term memory is preserved after elimination of synaptic connections. This is confirmed by the fact that the proteins involved in memory formation, including the transfer of genetic information through synapses between neurons (Arc protein), originate from transposons. Long non-coding RNAs and microRNAs also originate from transposons; their role in memory consolidation has been described. Pathological activation of transposable elements is a likely cause of neurodegenerative diseases with memory impairment. Analysis of the scientific literature allowed us to identify changes in the expression of 40 microRNAs derived from transposons in Alzheimer's disease. For 24 of these microRNAs, the mechanisms of regulation of genes involved in the functioning of the brain have been described. It has been suggested that the microRNAs we identified could become potential tools for regulating transposon activity in the brain in order to improve memory.

Key words: long noncoding RNAs; long-term memory; miRNAs; retroelements; transposons; epigenetic factors.

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Гипотеза взаимосвязи эпигенетических факторов с транспозонами в формировании памяти

Р.Н. Мустафин

Башкирский государственный медицинский университет, Уфа, Россия
ruji79@mail.ru

Аннотация. В обзорной статье описана гипотеза, согласно которой драйверами эпигенетической регуляции в формировании памяти являются мобильные генетические элементы, влияющие на экспрессию специфических генов в головном мозге. В подтверждение приведены результаты научных исследований о закономерной активации транспозонов в нейрональных стволовых клетках при дифференцировке нейронов. Данные процессы происходят в зоне нейрогенеза – зубчатой извилине гиппокампа, где определяются наибольшая активность мобильных генетических элементов и их инсерции в локусы вблизи генов, экспрессируемых нейронами с их активацией. В экспериментах по изменению активности ацетилтрансферазы гистонов, ингибированию ДНК-метилтрансферазы и обратной транскриптазы было показано вовлечение эпигенетических факторов и ретроэлементов в механизмы формирования памяти. В то же время в ряде работ на разных животных продемонстрировано сохранение долговременной памяти без участия синаптической пластичности. Полученные данные позволяют предположить, что транспозоны, являющиеся высокочувствительными сенсорами генома к различным средовым и внутренним воздействиям, формируют память на уровне ядерного кодирования. Это отражается в изменении синаптической пластичности, чем можно объяснить сохранение долговременной памяти после устранения синаптических связей у животных. Подтверждением слу-

жат факты происхождения от мобильных генетических элементов белков, непосредственно участвующих в формировании памяти, в том числе в передаче генетической информации через синапсы между нейронами (белок Arc). Транспозоны – источники длинных некодирующих РНК и микроРНК, роль которых в консолидации памяти описана. Патологическая активация мобильных генетических элементов является вероятной причиной нейродегенеративных болезней с нарушением памяти. Анализ научной литературы позволил нам обнаружить данные об изменениях экспрессии 40 микроРНК, произошедших от транспозонов, при болезни Альцгеймера. Для 24 из этих микроРНК описаны механизмы регуляции генов, участвующих в функционировании головного мозга. Сделано предположение, что установленные нами микроРНК могли бы стать потенциальными инструментами для регуляции активности транспозонов в головном мозге с целью улучшения памяти.

Ключевые слова: длинные некодирующие РНК; долговременная память; микроРНК; ретроэлементы; транспозоны; эпигенетические факторы.

Introduction

Memory is defined as the storage and use of received information in the brain during adaptation to the environment during the life of the organism. Memory includes the processes of encoding, consolidation, storage of information, and recollection. The molecular and cellular mechanisms of memory formation are logically interpreted by the transmission of nerve impulses between the synapses of many neurons. Most modern researchers explain the processes of memory formation by synaptic plasticity (SP) – the possibility of differential changes in the strength of neuronal transmission through certain synapses (with the weakening of some and strengthening of other connections between neurons) (Ortega-de San Luis, Ryan, 2022). There are four levels of memory study: psychological, neurophysiological, biochemical and cybernetic. According to the neurophysiological concept, memory is divided into short-term and long-term memory (LTM). Most modern neurophysiological theories boil down to the role of synaptic plasticity in the formation of LTM, which is closely related to the biochemical theory, since the electrochemical reaction of the formation of a nerve impulse turns into the biochemical process of the formation of new proteins (Munin, Olenko, 2022).

Reconsideration of the classical hypothesis of synaptic plasticity is necessary in connection with the growing evidence of memory retention without the participation of synaptic plasticity. The results of the first experiments in this area were published in 1984. Preservation of odor avoidance memory formed at the caterpillar stage was revealed in mature *Manduca sexta* moths after metamorphosis with reorganization of synapses (Levine, 1984). The memory of recognizing a textured surface to determine the presence of food was retained in planarians after head removal and subsequent brain regeneration (Shomrat, Levin, 2013). In a coculture of motor and sensory neurons from the sea hare *Aplysia*, memory for training with interval serotonin pulses persisted after its apparent elimination by anti-mnemonic drugs that erase learning-associated synaptic growth (Chen et al., 2014). In experiments on mice, the restoration of fear memory was determined when engram cells were reactivated in the absence of synaptic changes (after administration of the protein synthesis inhibitor anisomycin) (Ryan T.J. et al., 2015).

Various genes are involved in the formation of LTM, the most famous of which is *CREB* (cAMP-responsive element binding protein). Mutations in the *CREB* gene cause memory deficits in mice (Hegde, Smith, 2019). The *CREB* gene product, together with glucocorticoid receptors, is involved in the intracellular mechanisms of the influence of glucocorticoids on LTM formation in the hippocampus (Buurstede et al., 2022).

Experiments on Drosophila demonstrated the role of the beta-catenin gene (*CTNNB1*) in the consolidation of LTM due to its effect on Wnt signaling pathways (Tan Y. et al., 2013). Systematic reviews of data accumulated in the scientific literature have shown a stimulating effect of transcription factors on memory development, which are protein products of the expression of the following genes: *NF-κB* (Kaltschmidt B., Kaltschmidt C., 2015), *Zif268*, *XBP1*, *Srf*, *Npas4*, *Foxp1*, *Crtc1*, *c-Rel* (Hegde, Smith, 2019). In addition to the genes necessary for memory consolidation, which also include *NR2B* (encodes a subunit of the inotropic glutamate receptor N-methyl-d-aspartate), memory suppressor genes, which include *AIM2*, *ATF4*, *BChE*, *Bec1*, *CCR5*, *Cdk5*, *Crtl1*, *Diap1*, *Dicer1*, *DFF45*, *GABAaB3*, *GABAARα4*, *Gabra 4*, *Galectin-3*, *GAT1*, *QR2*, *Np65*, *Hcn1*, *Hdac2*, *Mef2*, *Kvβ1.1*, *PDE1b*, *Paip2a*, *Pkr*, *GCN2*, *IRS2*, *RGS14*, *RARalpha*, *p75NTR*, *PDE4A*, *Ogg1*, *PERK*, *RPTPsigma*, *Piwi1*, *Piwi2*, *S100b*, *TLCN*, *Pde4d/8b*, *11b-HSD1*, are important in the regulation of LTM (Noyes et al., 2021).

The results obtained indicate the presence of other mechanisms for maintaining LTM, which are realized in the form of synaptic plasticity. Most likely, memory is consolidated at the level of nuclear DNA under the driver influence of transposable elements (TEs), which rearrange the chromatin structure upon their activation, and are also integrated into specific loci during neuronal differentiation (Perrat et al., 2013; Upton et al., 2015). Chromatin remodeling under the influence of epigenetic modifications is necessary for the preservation and maintenance of memory, since it is reflected in changes in gene regulation in the brain. Epigenetic factors include cytosine methylation at CpG dinucleotides, chromatin modifications, and non-coding RNAs (ncRNAs), all of which are involved in long-term memory formation (Lipsky, 2013). The role of epigenetic factors in memory formation has been proven in experiments. Exposure to a DNA methyltransferase inhibitor destroyed fully consolidated fear memory one month after its onset (Miller et al., 2010).

Enhancing histone acetylation by manipulating the activity of a specific isoform of histone acetyltransferase (HAT) in

neurons significantly reduced memory consolidation (Jarome, Lubin, 2014). The formation of LTM is influenced by specific histone modifications: H2BK120ub, H3K9me2, H3K36me3, H3K27me3, H3K9me3, H3K4me3, H3K14ac, H3K9ac (Hegde, Smith, 2019).

At the same time, DNA methylation and chromatin reorganization enzymes interact with microRNAs (Mustafin, Khusnutdinova, 2017), which can also serve as guides recognizing complementary genome sequences in the mechanism of RNA-dependent DNA methylation (Chalertpet et al., 2019). This phenomenon suggests the role of TEs as drivers of epigenetic regulation of memory, since transposons are important sources for the emergence of microRNA genes (Wei et al., 2016). The role for TEs in regulation of neuronal function in humans was suggested in a 2022 review (Chesnokova et al., 2022). Although TEs cannot be the drivers of all epigenetic changes associated with mnestic processes, they are the evolutionary sources of many microRNAs (Wei et al., 2016), most long non-coding RNAs (lncRNAs) (Johnson, Guigo, 2014), and can themselves be transcribed directly into lncRNAs (Lu X. et al., 2014; Honson, Macfarlan, 2018). Consequently, transposons, to one degree or another, participate in most epigenetic and gene networks regulating genome functioning. In addition, TEs themselves are under the control of epigenetic changes, in part due to the mutual regulatory effects of microRNAs derived from them (Mustafin, Khusnutdinova, 2017).

Epigenetic regulation of transposons involves KRAB zinc finger proteins via heterochromatin initiation complexes, which modify histones and alter DNA methylation (Wolf et al., 2015). More than half of the binding regions for the PLZF (Promyelocytic Leukemia Zinc Finger) protein, a member of the Krüppel-type zinc finger family, are located within the LINE1 element genes (Lapp, Hunter, 2016). The SOX2 and HDAC1 control LINE1 activity by binding to the transcriptional repressor methyl-CpG-linked protein-2 (MeCP2). There are many other ways to regulate transposon activity, which include the APOBEC3 (Mager, Stoye, 2014), APOBEC1, ERCC, TREX1, RB1, HELLS, MEGP2 (Rodic, Burns, 2013), SIRT6 proteins (Van Meter et al., 2014).

In the MDTE DB database, 661 human microRNAs derived from TEs have been published (Wei et al., 2016). In neuronal stem cells (NSCs), transposon activation promotes genomic mosaicism of maturing neurons, which is necessary for their differentiation (Muotri et al., 2005). These changes are found in the zone of neurogenesis, the dentate gyrus of the hippocampus, not only in experimental animals, but also in humans (Coufal et al., 2009; Baillie et al., 2011; Kurnosov et al., 2015). In this case, TEs are inserted into genes or near genes involved in the functioning of neurons (Upton et al., 2015), and the hippocampus plays a key role in learning and memory formation (Zhang H. et al., 2021).

TEs can be activated under the influence of environmental factors, the signals of which enter the brain through neural networks, since transposons are highly sensitive sensors of environmental and internal changes (Mustafin, Khusnutdinova, 2019). Transposons are regions of the genome that move within the genome using the mechanism of "cut and paste" (DNA transposons) and "copy and paste" (retroelements – RE). REs may contain long terminal repeats (LTR-REs) or not contain them (non-LTR-REs). In humans, the latter include autonomous REs (LINEs – long interspersed nuclear elements) and non-autonomous ones (SINEs – short interspersed nuclear elements, SVA – SINE-VNTR-Alu elements) (Mustafin, Khusnutdinova, 2017).

Most lncRNAs, like microRNAs, are derived from transposons. On average, 41 % of lncRNA exons contain RE sequences, and 83 % of them contain at least one transposon fragment (Johnson, Guigo, 2014; Wei et al., 2016). Moreover, LINE1 transcripts themselves function as lncRNAs, interacting with specific regions of chromatin and regulating gene expression (Honson, Macfarlan, 2018), and LTR-REs serve as genes for many lncRNAs (Lu X. et al., 2014). Therefore, the participation of ncRNAs in memory storage indicates the importance of transposons in these processes.

The role of non-coding RNAs in memory formation

The tissue specificity of lncRNAs exceeds that of proteins. In the regulation of stem cell differentiation, they interact with REs (Ramsay et al., 2017). lncRNAs are formed from intergenic regions of eukaryotic genomes, characterized by tissue-specific transcription, from overlapping and antisense patterns relative to adjacent genes, which they regulate (Arendt et al., 2017). This allows them to determine a variety of cellular phenotypes, especially in the brain (Lapp, Hunter, 2016), which may reflect the role of transposons in these processes (Coufal et al., 2009; Baillie et al., 2011). RNA sequencing analysis with induction of long-term potentiation (LTP) in the dentate gyrus of rats after high-frequency stimulation of the perforant pathway showed a positive, pronounced correlation of the dynamic expression of lncRNAs with REs and protein-coding genes (Maag et al., 2015).

A number of scientific works have shown the role of specific lncRNAs in memory consolidation. Experiments on rodents revealed that lncRNA NEAT1 is an epigenetic suppressor of LTM formation in the hippocampus (Butler et al., 2019). Increased expression of the lncRNA SLAMR was detected in hippocampal neurons under the influence of contextually conditioned fear. SLAMR is transported to dendrites via the molecular motor KIF5C and is recruited to the synapse in response to stimulation. SLAMR modulates the activity of the CaMKIIα protein, which plays an important role in synaptic plasticity in synaptoneurosomes (Espadas et al., 2023). lncRNA Carip also interacts with the CaMKII protein, which controls the phosphorylation of AMPA and NMDA receptors in the hippocampus, affecting spatial memory. In the absence of Carip, synaptic plasticity dysfunction occurs in CA3-CA1 in the hippocampus, indicating the role of this lncRNA in memory regulation (Cui et al., 2022). Since many lncRNAs are expressed in the brain, they may regulate genes of LTM (Samaddar, Bnerjee, 2021).

At least 70 % of human miRNAs are expressed in the brain, with a specific miRNA activation pattern for each region (Chen, Qin, 2015). In hippocampal neurons, induction

of Dicer by the BDNF protein leads to increased synthesis of miR-7a, -7b, -7f, -9, -107, -124a, -125b, -132, -134, -143, -375, which are involved in the regulation of memory (Leal et al., 2014). A systematic review of the scientific literature showed an increase in the expression of miR-124, miR-134, miR-206, as well as a decrease in the expression of miR-9-3p, miR-92, miR-195 and the miR-183/96/182 cluster during LTM consolidation (Grinkevich, 2020). miR-124 and miR-12 promote the formation of the early phase of long-term memory (Michely et al., 2017). Because miRNAs play a role in normal memory formation, their abnormal expression may play a role in the development of neurodegenerative memory-impairing diseases such as Alzheimer's disease (AD). A systematic review of the scientific literature conducted in 2019 showed the post-transcriptional regulatory influence of specific microRNAs on the mRNA of genes involved in the pathogenesis of AD. It was revealed that miR-17, -655, -644, -323-3p, -153, -147, -20a bind to APP protein mRNA. miR-1306, -451, -181, -144, -107, -103, -9 have an inhibitory effect on α-secretase ADAM10; miR-101, -107, -384, -339-5p, -200b, -195, -186, -135a, -29a, -29b-1, -29c inhibit β-secretase BACE1 (Patel et al., 2019).

The role of retroelements in the consolidation of long-term memory

The role of REs in the formation of long-term memory is evidenced by the results of experimental work of independent researchers. Thus, by inhibiting LINE1 in the hippocampus of mice, the role of REs in memory consolidation resulting from genomic mosaicism was determined. To do this, the animals were placed on an illuminated side, after which they were allowed to move to the dark side of the chamber, where they were exposed to current. The learning memory was reflected in an increase in mouse latency when moving to the dark side of the chamber. Long-term memory was significantly impaired 72 hours after hippocampal administration of the reverse transcriptase inhibitor lamivudine (Bachiller et al., 2017). Another study of context-dependent fear memory, in addition to demonstrating significant suppression of long-term memory following lamivudine administration, identified LINE1 expression in the hippocampus and prefrontal cortex during fear memory using quantitative real-time PCR of hippocampal and prefrontal cortex samples (Zhang W.J. et al., 2021). A significant number of TEs transpositions (more than 200 per cell) in memory-related neurons have also been identified in the Drosophila brain (Perrat et al., 2013). Since the results of many of the studies cited in the review reflect correlational relationships and require more direct confirmation of the effect of transposons on memory, such as single-cell sequencing of the hippocampal region and specific areas of the cerebral cortex before and after long-term memory training.

According to data from the ENCODE and FANTOM consortia, transposon activity depends on the cell type and affects the expression of neighboring genes. TEs are of greatest importance in brain function regulation, providing adaptive functions of the central nervous system. In response to the effects of steroids, epigenetic and environmental factors, they change the functioning of neurotransmitter systems to adapt to changing environmental conditions, including LTM formation (Lapp, Hunter, 2016). Activated REs play a regulatory role not only for NSCs, but also in the late phase of neuronal differentiation (Muotri et al., 2010), controlling the specific pattern of gene expression in neurons located in certain areas of the brain, due to which memory is formed (Singer et al., 2010). In the mouse hippocampus, SINE expression profiles are cell type specific. In response to brief exposure to a novel stimulus, SINEs are activated in dentate granule neurons over a time course similar to that of protein-coding genes (Linker et al., 2020).

LTR-REs play an important role in the development of long-term memory. The protein product of the HERV *env* gene activates BDNF (brain-derived neurotrophic factor) (Huang et al., 2011), which is involved in synaptic transmission and LTP in the hippocampus and other brain regions for the formation of various forms of memory (Leal et al., 2014). In evolution, the domestication of LTR-REs led to the formation of genes that are key to long-term memory. According to computer analysis (Campillos et al., 2006), confirmed by phylogenetic studies (Ashley et al., 2018; Pastuzyn et al., 2018), the *Arc* gene (Activity-regulated cytoskeleton-associated protein) in humans originated from ERV Ty3/gypsy. The Arc protein regulates synaptic plasticity in the control of signaling networks during memory consolidation. *Arc* gene transcripts are transported to dendrite synapses, where they are synthesized into protein on ribosomes. Arc forms a capsid that encapsulates its own mRNAs. The resulting virus-like structures are loaded into extracellular vesicles and transported to neurons, transmitting genetic information and regulatory signals through neural networks, which is necessary for the formation of LTM (Ashley et al., 2018; Pastuzyn et al., 2018).

The Prp8 protein, which is a component of the eukaryotic spliceosome, evolved from ERV reverse transcriptase (Dlakić, Mushegian, 2011). Experiments on Drosophila demonstrated the key role of Prp8 in controlling the expression of the neuropeptide FMRFa in neurons (Cobeta et al., 2018). The TERT protein, derived from retroelement reverse transcriptase (Kopera et al., 2011), regulates spatial memory formation by modulating neuronal development in the hippocampus (Zhou et al., 2017). The Gag ERV protein gave rise to the PEG10 protein, which interacts with ATXN2 and ATXN10 in stress granules and extracellular vesicles and regulates neuronal migration during LTM formation (Pandya et al., 2021). In evolution, Gag also became the source of the CCHC type of zinc finger protein (encoded by the *SIRH11/ZCCHC16* gene). Deletion of the *SIRH11/ZCCHC16* gene in mice causes abnormal behavior associated with cognitive abilities, including working memory (Kaneko-Ishino, Ishino, 2016). The *Gag* gene was the origin of the *RTL1/PEG11* gene expressed in the brain. Mice with knockout of the paternal allele (Rtl1m+/p–) showed decreased memory (Chou et al., 2022). The data obtained on the role of ERV-derived proteins in LTM formation indicate the potential participation of ERVs themselves in these processes.

Thus, the strength of the hypothesis of the role of transposons in the formation of long-term memory is the presence of direct experimental evidence of the participation of REs in

these processes (Singer et al., 2010; Huang et al., 2011; Perrat et al., 2013; Leal et al., 2014; Lapp, Hunter, 2016; Bachiller et al., 2017; Zhang W.J. et al., 2021). There is also indirect evidence of the importance of mobile genetic elements in the mechanisms of long-term memory, since REs are the evolutionary sources of proteins involved in the formation of memory, such as Arc (Campillos et al., 2006; Ashley et al., 2018; Pastuzyn et al., 2018), Prp8 (Dlakić, Mushegian, 2011; Cobeta et al., 2018), TERT (Kopera et al., 2011; Zhou et al., 2017), PEG10 (Pandya et al., 2021), CCHC type zinc finger protein (Kaneko-Ishino, Ishino, 2016).

TEs are also sources of microRNAs (Wei et al., 2016) and long ncRNAs (Johnson, Guigo, 2014), which are actively involved in the epigenetic regulation of LTM. Therefore, the strength of the proposed hypothesis is the explanation of the mechanisms of influence of environmental stimuli on epigenetic factors, since in these processes REs are effective mediators, sensitive not only to stress, but also to many external and internal factors, perceiving information for the adaptation of the body (Mustafin, Khusnutdinova, 2019), which corresponds to the definition of memory (Ortega-de San Luis, Ryan, 2022). Moreover, the ability of REs to insert into new loci of the genome, thereby changing the expression of specific genes involved in the formation of long-term memory, in contrast to the synaptic plasticity hypothesis, explains long-term consolidation at the genome level (Perrat et al., 2013; Bachiller et al., 2017; Zhang W.J. et al., 2021).

The proposed hypothesis is also supported by the high rate of information consolidation at the genome level (compared to the possible influence of the potential on protein synthesis on ribosomes) due to the activation of REs, since TEs are involved in many gene and epigenetic networks (due to the presence of sequences complementary to non-coding RNAs derived from TEs). Therefore, environmental stimuli that activate TEs can quickly affect changes in gene networks, which is sufficient for the formation of LTM.

A possible counterargument to the role of transposons in memory formation can be the fact of activation of REs during aging, which is characterized by a decline in cognitive functions and memory. However, a systematic review of the scientific literature showed that the cause of aging is a pathological activation of REs (Mustafin, Khusnutdinova, 2018a), while for ontogenesis, starting from the division of the zygote and until reaching the sexually mature state of the organism, species-specific activation of strictly defined transposons occurs, including in the brain (Mustafin, Khusnutdinova, 2018b). This statement is supported by pathological activation of REs in diseases associated with old age, characterized by impaired long-term memory.

Relationship between transposons and microRNAs in memory disorders

Prospects for studying the relationship of TEs with epigenetic factors in the formation of LTM in health and disease are associated with the possibility of correcting disorders, since epigenetic changes are reversible. Although TE expression is required for normal memory consolidation, their pathological activation is a factor in the development of neurodegenerative diseases. Experiments on mice modeled for AD showed impairment of long-term memory due to derepression of REs (El Hajjar et al., 2019). G-quadruplex derived from evolutionarily conserved LINE1 suppresses gene expression in Alzheimer's disease neurons (Hanna et al., 2021). In the mouse brain, tau proteins activate ERVs, increasing their DNA copy numbers (Ramirez et al., 2022), and in patients with Alzheimer's disease, decondensation of heterochromatin and reduction in piwi and piRNA levels activate HERVs (Sun W. et al., 2018), LINE1 and Alu (Grundman et al., 2021).

Since microRNAs are also characterized by changes in expression during normal memory formation (Leal et al., 2014; Chen, Qin, 2015; Michely et al., 2017; Grinkevich, 2020) and in Alzheimer's disease (Patel et al., 2019), these changes may be associated with pathological TE activation. To confirm this assumption, we analyzed the scientific literature on the relationship between microRNAs and TEs in Alzheimer's disease. For this purpose, we studied the results of studies on changes in the expression of microRNAs derived from mobile genetic elements (according to the MDTE database (Wei et al., 2016)) in Alzheimer's disease. As a result, 40 such microRNAs were discovered, which indicate the role of microRNAs interconnected (due to complementarity of nucleotide sequences) with TEs in the mechanisms of memory formation in humans under normal and pathological conditions. For 24 of these miRNAs, functional significance in the brain was described (see the Table).

Conclusion

Investigation of the role of epigenetic factors in normal and pathological long-term memory formation is promising due to the reversibility of changes occurring under their influence and the possibility of influencing them with the help of microRNAs. The most likely drivers of epigenetic regulation of genes during memory formation are TEs – highly sensitive genome sensors to environmental and internal influences. This is evidenced by the preservation of long-term memory with the complete elimination of synaptic connections. TEs consolidate memory at the level of nuclear DNA due to a programmed pattern of their activation and transposition. An analysis of the scientific literature made it possible to find evidence of the role of TEs, lncRNAs and microRNAs interconnected with them in the formation of memory in health and disease. In Alzheimer's disease, changes in the expression of 40 microRNAs derived from TEs were determined, the majority of which originate from REs (24 microRNAs – from LINEs, 7 – from SINEs, 5 – from ERVs).

It can be assumed that in the future the identified microRNAs may become objects and tools for regulating the activity of TEs in the brain. The proposed hypothesis of the role of REs in the formation of LTM explains the missing links in the theory of synaptic plasticity, since activated transposons form insertions in specific genomic loci that change the expression of genes involved in the development of memory, which explains the consolidation of LTM at the level of nuclear coding.

Association of transposon-derived microRNAs with Alzheimer's disease

microRNA	microRNA source	Changes in microRNA expression in Alzheimer's disease (author) (↑ – increase, ↓ – decrease)	The role of microRNAs in the brain (author)
miR-1202	LINE1	↑ (Henriques et al., 2020)	Inhibits *Rab1a* and TLR4/NFκB signaling pathways (Song et al., 2020)
miR-1246	ERVL	↑ (Guo et al., 2017)	NA
miR-1271	LINE2	↓ (Majumder et al., 2021)	Interacts with the mRNA of the tyrosine kinase receptors ALK and RYK (Majumder et al., 2021)
miR-151	LINE2	↑ (Satoh et al., 2015)	Regulates memory in the hippocampus (Xu X.F. et al., 2019; Ryan B. et al., 2017)
miR-192	LINE2	↓ (Qin et al., 2022)	Affects the TGF-β1 signaling pathway (Tang et al., 2019)
miR-211	LINE2	↑ (Sierksma et al., 2018; Li et al., 2021)	Regulates migration and differentiation of neurons (Mainigi et al., 2016)
miR-224	MER135	↓ (Sun X. et al., 2023)	Inhibits the NLRP3 inflammasome (Sun X. et al., 2023), regulates NPAS4, is involved in long-term memory (Bersten et al., 2014)
miR-28	LINE 2	↑ (Hong et al., 2017; Zhao et al., 2020)	NA
miR-31	LINE2	↓ (Barros-Viegas et al., 2020)	Regulates LTP (Parsons et al., 2012)
miR-3199	LINE2	↓ (Sun C. et al., 2021)	Participates in the regulation of beta-amyloid expression (Sun C. et al., 2021)
miR-320c	LINE2	↑ (Raheja et al., 2018; Boese et al., 2016)	NA
miR-3200	ERV-L	↓ (Satoh et al., 2015)	NA
miR-325	LINE2	↓ (Barak et al., 2013)	Inhibits tomosyn protein expression (impairs synaptic plasticity in the hippocampus (Barak et al., 2013))
miR-326	hAT-Tip100	↑ (Cai et al., 2017)	Regulates genes involved in synaptic plasticity (Cohen et al., 2014); in response to BDNF, it reduces Arc expression (Wibrand et al., 2012)
miR-335	SINE	↑ (Bottero, Potashkin, 2019)	Modulates synaptic plasticity and spatial memory in the hippocampus (Capitano et al., 2017)
miR-340	TcMar	↓ (Tan X. et al., 2020)	
miR-342	SINE	↓ (Dakterzada et al., 2021)	Regulates the enzyme that breaks down amyloid precursor protein (BACE1) (Dong et al., 2022)
miR-3646	SINE	↑ (Lu L. et al., 2021)	NA
miR-378a	SINE	↑ (Dong et al., 2021)	Inhibits the *EZH2* gene, regulating the production of pro-inflammatory cytokines (Weng et al., 2023)
miR-384	LINE/Dong-R4	↑ (Samadian et al., 2021)	Is involved in LTP maintaining (Gu et al., 2015)
miR-4286	LTR/ERVL	↓ (Henriques et al., 2020)	NA
miR-4422	LTR/Gypsy	↓ (Hajjri et al., 2020)	NA
miR-4487	LINE1	↓ (Hu et al., 2018)	Suppresses beta-amyloid-induced apoptosis (Hu et al., 2018)
miR-4504	LINE1	↑ (Eysert et al., 2021)	Inhibits the APP-interacting *FERMT2* gene (Eysert et al., 2021)
miR-4772	LINE1	↓ (Lugli et al., 2015)	NA
miR-495	ERVL	↑ (Yuen et al., 2021)	Participates in memory formation in the hippocampus (Puig-Parnau et al., 2020)
miR-502	LINE2	↓ (Satoh et al., 2015)	NA
miR-511	LINE1	↓ (Wang et al., 2023)	Regulates neuronal differentiation by inhibiting the *FKBP5* gene (Zheng et al., 2016)
miR-517	SINE/Alu	↑ (Schipper et al., 2007)	NA
miR-545	LINE2	↓ (Cosín-Tomás et al., 2017)	NA
miR-566	SINE/Alu	↑ (Yaqub et al., 2023)	NA
miR-576	LINE1	↓ (Liu et al., 2014; Xu X. et al., 2022)	NA
miR-582	LINE/CR1	↓ (Eysert et al., 2021)	Suppresses *FERMT2* gene expression (Eysert et al., 2021)

Table (end)

microRNA	microRNA source	Changes in microRNA expression in Alzheimer's disease (author) (↑ – increase, ↓ – decrease)	The role of microRNAs in the brain (author)
miR-603	TcMar	↑ (Zhang C. et al., 2016)	NA
miR-6087	LINE1	↓ (Lau et al., 2013)	NA
miR-619	LINE1	↓ (Baek et al., 2021)	Regulates circadian rhythm genes *PPP1CB*, *PPP1CC*, *CREBBP*, *HELZ2*, *NCOA1*, *TBL1X* (Baek et al., 2021)
miR-659	LINE2	↓ (Lugli et al., 2015)	Inhibits the progranulin gene (*GRN*) (Pisopo et al., 2016)
miR-664	LINE1	↓ (Schonrock et al., 2010)	Binds to the 3'UTR of the *NMDAR1* gene mRNA, which stimulates GnRH (Ju et al., 2019)
miR-708	LINE2	↓ (Rahman et al., 2020; Di Palo et al., 2022)	Regulates neuronatin synthesis (Vatsa et al., 2019)
miR-885	SINE/MIR	↓ (Tan L. et al., 2014)	Inhibits *KREMEN1* gene expression (Pan et al., 2022)

Note. NA – no data available.

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Generation and analysis of mouse embryonic stem cells with knockout of the *Mcph1* (microcephalin) gene

A.M. Yunusova [1], A.V. Smirnov [1], T.A. Shnaider [1], I.E. Pristyazhnuk [1], S.Y. Korableva [2], N.R. Battulin [1, 2]

[1] Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
[2] Novosibirsk State University, Novosibirsk, Russia

anastasiajunusova@gmail.com

Abstract. Chromatin is not randomly distributed within the nucleus, but organized in a three-dimensional structure that plays a critical role in genome functions. Cohesin and condensins are conserved multi-subunit protein complexes that participate in mammalian genome organization by extruding chromatin loops. The fine temporal regulation of these complexes is facilitated by a number of other proteins, one of which is microcephalin (Mcph1). Mcph1 prevents condensin II from associating with chromatin through interphase. Loss of Mcph1 induces chromosome hypercondensation; it is not clear to what extent this reorganization affects gene expression. In this study, we generated several mouse embryonic stem cell (mESC) lines with knockout of the *Mcph1* gene and analyzed their gene expression profile. Gene Ontology analyses of differentially expressed genes (DEGs) after *Mcph1* knockout revealed gene categories related to general metabolism and olfactory receptor function but not to cell cycle control previously described for Mcph1. We did not find a correlation between the DEGs and their frequency of lamina association. Thus, this evidence questions the hypothesis that Mcph1 knockout-mediated chromatin reorganization governs gene expression in mESCs. Among the negative effects of *Mcph1* knockout, we observed numerous chromosomal aberrations, including micronucleus formation and chromosome fusion. This confirms the role of Mcph1 in maintaining genome integrity described previously. In our opinion, dysfunction of Mcph1 may be a kind of "Rosetta stone" for deciphering the function of condensin II in the interphase nucleus. Thus, the cell lines with knocked-out *Mcph1* can be used to further study the influence of chromatin structural proteins on gene expression.
Key words: Mcph1/microcephalin; chromosome condensation; mESCs; gene expression analysis.

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Получение и характеристика линий эмбриональных стволовых клеток мыши с нокаутом гена *Mcph1* (микроцефалин)

А.М. Юнусова [1], А.В. Смирнов [1], Т.А. Шнайдер [1], И.Е. Пристяжнюк [1], С.Ю. Кораблёва [2], Н.Р. Баттулин [1, 2]

[1] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Новосибирский национальный исследовательский государственный университет, Новосибирск, Россия

anastasiajunusova@gmail.com

Аннотация. Хроматин в ядре клетки распределен не хаотично, а имеет организованную структуру, которая оказывает прямое влияние на функционирование генома. Одними из основных архитектурных белков хроматина в клетках млекопитающих являются консервативные мультисубъединичные белковые комплексы: когезин и конденсины. Эти комплексы способны протягивать петли хроматина, опосредуя контакты между удаленными участками ДНК. Тонкая временная регуляция их активности осуществляется рядом других белков, один из которых – микроцефалин (Mcph1). Mcph1 препятствует взаимодействию конденсина II с хроматином в интерфазе. При нарушении его функции наблюдается масштабная реорганизация хроматина, вызванная аномальной загрузкой конденсина II. Как это сказывается на экспрессии генов, до сих пор неизвестно. В данном исследовании мы создали несколько линий эмбриональных стволовых клеток мыши с нокаутом гена *Mcph1*, охарактеризовали их и проанализировали профиль экспрессии генов. Аннотация дифференциально экспрессирующихся генов в терминах генной онтологии выявила категории генов, относящиеся к общему метаболиз-

му и функционированию обонятельных рецепторов, но не к регуляции клеточного цикла, описанной ранее для Mcph1. Мы также не обнаружили корреляции между генами, изменившими свою транскрипционную активность после нокаута *Mcph1*, и вероятностью их локализации на ядерной ламине. Этот результат ставит под сомнение гипотезу о влиянии опосредованной нокаутом *Mcph1* архитектуры хроматина на экспрессию генов. Среди негативных эффектов нокаута *Mcph1* мы наблюдали множественные хромосомные аберрации, включая нарушения сегрегации хромосом с образованием микроядер, а также слияние хромосом. Это подтверждает описанную в предыдущих исследованиях роль белка Mcph1 в поддержании целостности структуры генома. Мы полагаем, что нокаут *Mcph1* может оказаться своеобразным «розеттским камнем», способным расшифровать функции конденсина II в интерфазном ядре. Полученные нами линии эмбриональных стволовых клеток с нокаутом гена *Mcph1* могут быть использованы для дальнейшего изучения влияния структурных белков хроматина на экспрессию генов.

Ключевые слова: Mcph1 (микроцефалин); конденсация хромосом; ЭС клетки мыши; транскриптомный анализ.

Introduction

The three-dimensional organization of chromatin plays a crucial role in maintaining genome stability and regulating key cellular processes such as DNA replication, DNA repair, and gene expression (Marchal et al., 2019; Stadhouders et al., 2019; Sanders et al., 2020). Interphase chromosomes are decondensed and distributed all over the nucleus. Contacts between distant genomic regions are important in the regulation of gene expression and mediated by CTCF and cohesin complexes (SMC family of ATPases) (Dixon et al., 2012; Rao et al., 2014) (Fig. 1). The transition from interphase to mitosis leads to significant chromatin structure changes: chromosomes become highly compacted due to the loading of condensin complexes – other members of the SMC protein family (Earnshaw, Laemmli, 1983; Naumova et al., 2013). Condensin II builds large regular chromatin loops early in mitosis forming helically arranged axial scaffold, whereas condensin I generates smaller nested loops inside the large loop and promotes the widening of the chromosomes. As mitosis progresses, outer loops grow and the number of loops per turn increases, promoting axial shortening of the chromosomes (Gibcus et al., 2018) (Fig. 1).

In recent years, interest in condensin complexes as motor proteins involved in establishing chromatin loops has greatly increased driven by advances in 3D genomics and super-resolution microscopy methods. However, many of their functions remain unclear. One of the most intriguing questions is the role of condensin II in the interphase nucleus (Wallace, Bosco, 2013). Unlike cytoplasmic condensin I, which interacts with chromatin only after nuclear envelope breakdown, condensin II is present in the nucleus throughout interphase (Hirota et al., 2004; Ono et al., 2004). Some studies suggest that condensin II loads coordinately with cohesin and transcription factor TFIIIC onto chromatin at the promoters of actively transcribed genes (Dowen et al., 2013; Yuen et al., 2017). Other studies indicated that condensin II does not play any significant role during interphase since the depletion of condensin II in non-dividing cells does not lead to changes in the spatial organization of the genome or gene transcriptional activity (Abdennur et al., 2018; Hoencamp et al., 2021). It is well established that condensin II's activity during interphase is blocked by microcephalin (Mcph1) (Trimborn et al., 2006; Yamashita et al., 2011; Houlard et al., 2021). Mcph1 is a multifunctional protein that also participates in DNA repair, cell

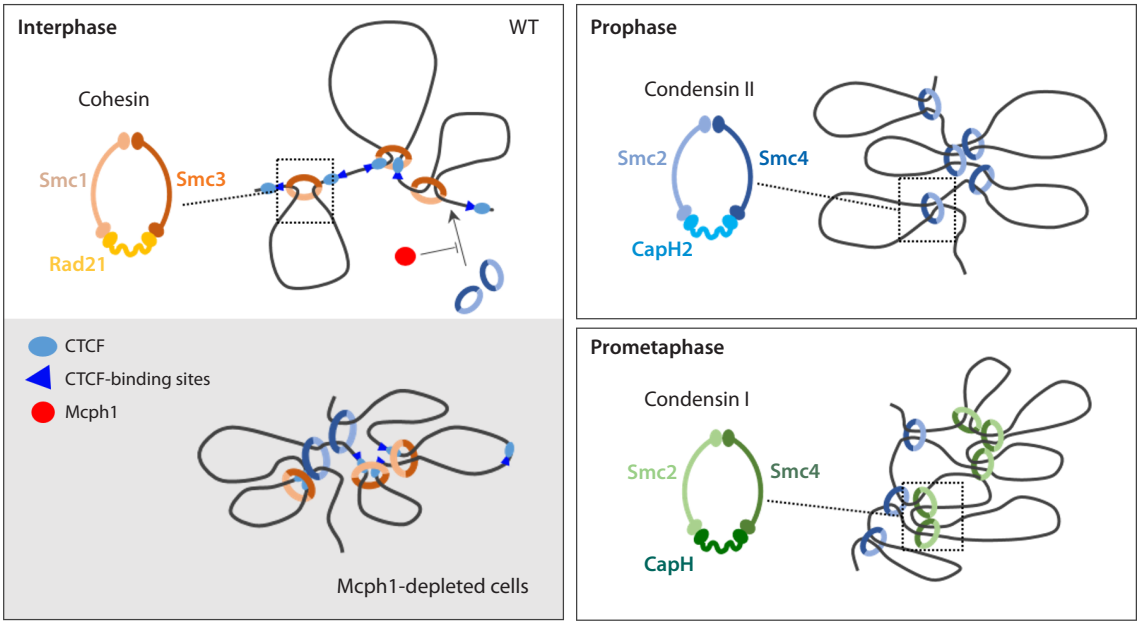

Fig. 1. DNA loop extrusion by SMC (Structural maintenance of chromosomes) complexes during cell-cycle progression in WT and Mcph1-depleted cells.

cycle control, apoptosis, and chromatin remodeling (reviewed by (Kristofova et al., 2022)). Mcph1 binds to condensin II through its short linear motif in the central domain thereby blocking the condensin II interaction with chromatin (Houlard et al., 2021). Disruption of Mcph1 function leads to chromosome condensation of interphase nuclei (Fig. 1). As a result, mutant cells acquire a unique phenotype characterized by prophase-like compacted chromosomes during interphase (Neitzel et al., 2002; Gruber et al., 2011).

It has been shown that in mouse embryonic stem cells (mESCs) *Mcph1* knockout leads to altered chromatin architecture by enhancing the mixing of A and B chromatin compartments. This is consistent with microscopic observations – highly condensed chromosomes become "individualized" in the interphase nuclei, while the chromocenters have disappeared (Houlard et al., 2021). Whether these chromatin state changes can affect gene expression is not clearly understood. To address this issue, we generated mESCs with stable *Mcph1* knockout and analyzed the changes in gene expression profiles.

Materials and methods

Mouse embryonic stem cells culture. All Δ*Mcph1* cell lines were generated from mouse ES cells (Rad21-miniIAA7-eGFP) previously established in our laboratory (Menzorov et al., 2019; Yunusova et al., 2021). Cells were cultured on the plates coated with a 1 % gelatin solution under 2i conditions, which ensures the pluripotency by specifically blocking the MAPK–ERK pathway (PD0325901, 1 μM) and glycogen synthase kinase 3 (CHIR99021, 3 μM) in DMEM (Thermo Fisher), supplemented with 7.5 % ES FBS (Gibco), 7.5 % KSR (Gibco), 1 mM L-glutamine (Sigma), NEAA (Gibco), 0.1 mM β-mercaptoethanol, LIF (1,000 U/ml, Polygen), and 1× penicillin/streptomycin (Capricorn Scientific). The growth medium was changed to a fresh one every day. Upon reaching appropriate confluence (70–80 %), the cells were passaged every 2–3 days.

Gene targeting of the *Mcph1* gene in mESCs. The sequences of guide RNAs were taken from the article (Houlard et al., 2021). gRNAs were cloned into the gRNA_cloning vector (Addgene, 41824). For exogenous Cas9 expression, the vector pCSDest2-2XNLS-SpCas9-WT-NLS-3XHA-NLS-TALentry (Addgene, 69232) was used. The plasmids were introduced into cells via electroporation (Neon Transfection System, Thermo Fisher Scientific, USA) as follows: for each 10 μl electroporation, 250,000 cells and 1 μg of total DNA (with an equimolar ratio of the two vectors) were used. Electroporation was performed following the manufacturer's protocol under conditions 6 and 10, previously determined as the most optimal for efficiency/survival ratio for mouse ESCs in our laboratory. After electroporation, cells were seeded into a 24-well plate in pre-warmed media without antibiotics. The next day cells were split into 10 cm dishes at low density. The medium was changed every 2–3 days. After single-cell clones were visible, a subset of clones was handpicked with pipette tips under the light microscope and transferred into a drop of trypsin/EDTA in each well of a 96-well plate, and then resuspended in growth medium. Upon reaching a confluent density, subclones were plated into two new 96-well plates (one for stock storage and one for PCR-genotyping).

For PCR-genotyping, cells were lysed in PBND buffer (10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45 % NP-40, and 0.45 % Tween 20, pH 8.3) containing 1 μg/μl proteinase K (NEB, USA) for an hour at 55 °C. After inactivation of proteinase K (95 °C, 10 minutes), 1 μl of lysate was used as a template for PCR amplification of the Cas9-target site. The target region included exon 2 of the *Mcph1* gene and was amplified using HS-Taq DNA Polymerase kit (Biolabmix, Russia) under the following conditions: 95 °C for 30 s, followed by 34 cycles of 95 °C – 10 s, 60 °C – 20 s, 72 °C – 1 min, and a final elongation at 72 °C for 5 minutes. The primers used were: Mcph1-del-F – ACCACATGCTTTGGCGTAGA and Mcph1-del-R – GCCAGACTCAAGTCTCCCAC. Amplified DNA fragments were separated on 2 % agarose gel. For selected subclones, amplicons were purified and their nucleotide sequence was determined by Sanger sequencing.

Protein detection by Western Blotting. Growth medium was discarded and cells were washed with PBS and scraped from the surface in the presence of RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, and 0.1 % SDS) containing the protease inhibitor cocktail [1x Complete ULTRA, 1x PhosSTOP (both from Roche, Switzerland), 5 mM NaF (Sigma-Aldrich, USA)]. After that, the cell lysates were sonicated by three 10 s pulses at 33–35 % power settings with UW 2070 (Bandelin electronics, Germany). The sonicated samples were centrifuged at 14,000 g for 20 min at 2 °C, frozen, and stored at –80 °C. The protein concentrations were quantified according to instruction's protocol by using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Equal amounts of the denatured total protein (20 μg) were separated on 10 % SDS-PAGE gel and then transferred onto the Immun-Blot PVDF membrane (Bio-Rad). After blocking in 5 % milk in TBST (50 mM Tris base, 150 mM NaCl, 0.05 % (v/v) Tween 20) for 2 h, the membranes were incubated with primary antibodies against Mcph1 protein (D38G5) Rabbit mAb #4120 (Cell Signaling Technology, USA) at a 1:1,000 dilution overnight at 4 °C. On the following day, after three washes with TBST buffer (10 min) membranes were incubated with horseradish peroxidase – conjugated secondary antibodies (Anti-rabbit IgG #7074, Cell Signaling Technology) for 2 h at room temperature. Detection was performed with Clarity™ (BioRAD, USA) and iBright™ FL1500 (Thermo Fisher Scientific, USA).

RNA extraction and transcriptome sequencing. The isolation of total RNA was performed using Trizol reagent (Sigma-Aldrich, MA, USA) following the manufacturer's instructions. The isolated RNA samples were resuspended in DEPC-treated water, then RNA concentration and quality were assessed by spectrophotometry and gel electrophoresis. Total RNA was sequenced on the BGISEQ-500 High-throughput Sequencing Platform (BGI, Beijing, China). The expression of RNA transcripts was quantified using Salmon (Patro et al., 2017). All analyses were performed using R Statistical Software (v4.3.2; R Core Team 2023). Raw counts were processed and normalized by Log2 fold change using tximport (https://github.com/thelovelab/tximport), genefilter (https://github.com/Bioconductor/genefilter), GenomicFeatures (https://github.com/Bioconductor/GenomicFeatures) and DESeq2 (https://github.com/thelovelab/DESeq2). Volcano plots were constructed using the EnhancedVolcano R package (https://

github.com/kevinblighe/EnhancedVolcano). The heat map was generated using the ComplexHeatmap (https://github.com/jokergoo/ComplexHeatmap). The gene ontology term enrichment analysis was carried out using the PANTHER server (https://www.pantherdb.org). The set of genes specifically expressed in mESCs with a base mean level ≥100 was used as a reference set.

To examine whether the differences in gene expression in Δ*Mcph1* cell lines were associated with the differences in lamina association, we used the LaminB1-DamID libraries from (Borsos et al., 2019). Next, we determined the DamID score in a 100 kb bin containing the coordinates of the transcription start sites of DEGs (Supplementary Material 1)[1]. To determine the correlation between the DamID score and the magnitude of the change in activity (log2FoldChange), the Pearson correlation coefficient was calculated for each gene.

Cell cycle analysis by flow cytometry. After trypsinization, cell pellets were washed with PBS and resuspended in cold 70 % ethanol for fixation overnight at 4 °C. The next day, the fixed cells were centrifuged and the fixative was thoroughly removed. The cell pellet was suspended in PI solution (1 % Triton X-100, 500 μg/ml propidium iodide, and 10 μg/ml RNase A in PBS) and incubated for 30 min at room temperature. After that, cell cycle distribution was analyzed using a BD FACS Aria flow cytometer (BD Biosciences, USA).

Chromosome spread analysis. Chromosome preparations were obtained following standard protocols (Matveeva et al., 2017). Briefly, cells were exposed to a 0.1 μg/ml colcemid (Merck, Germany) in growth medium for 3 h. After, cells were treated with 0.05 % Trypsin-EDTA solution (Capricorn Scientific GmbH, Germany), hypotonic solution (0.25 % KCl and 0.2 % sodium citrate) was added directly to the culture plates for 20 min at 37 °C. Then, cells were harvested, fixed with Carnoy fixative (3:1 methanol:glacial acetic acid) and dropped onto cold wet glass slides. Nuclear DNA was counterstained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, USA). Representative images were captured under a Carl Zeiss Axioscop 2 fluorescence microscope equipped with a CoolCube1 CCD-camera (Meta Systems, Altlussheim, Germany) at the Public Center for Microscopy SB RAS, Novosibirsk. The fraction of PLCs was determined after counting 130 to 230 nuclei in two replicas for each sample. The total length of all chromosomes in 15 metaphases for each sample was measured in arbitrary units (au) using ImageJ software.

Statistical analysis. Data analysis was performed using GraphPad Prism software package, employing two-sided Student's t test or Analysis of variance (ANOVA). Differences were considered statistically significant at $p < 0.05$.

Results

Generation and chromosome analysis of ΔMcph1 cells

Following the protocol described by (Houlard et al., 2021), we generated mESCs with a deletion of exon 2 of the *Mcph1* gene, consequently producing a gene knockout. Based on PCR genotyping, we selected 3 clones (#23_1, #70, #85) and confirmed the presence of the targeted deletion of exon 2 in clones #70 and #85 (Fig. 2*a*, *b*) by Sanger sequencing. We were unable to obtain satisfactory Sanger sequence data for clone #23_1 because of difficulties in resolving overlapping sequencing signal peaks of heterozygous deletions. However, this clone was included in further analysis. The absence of Mcph1 was confirmed by Western blotting for all subjected clones (Fig. 2*c*). For CRISPR/Cas9 off-targets analysis, we utilized NGS data from three *Mcph1*-knockout cell lines obtained previously by our group (unpublished data). We found no detectable off-target editing at the predicted sites (Supplementary Material 2).

It is known that dysfunction of Mcph1 is associated with an increased fraction of cells with prophase-like condensed (PLCs) chromosomes in interphase (Arroyo et al., 2017; Houlard et al., 2021) (Fig. 2*d*). For the mutant clones we calculated the proportion of PLCs, which amounted to over 20–30 %, significantly differing from that in the parental *Mcph1*+/+ cell line (4 %) (Fig. 2*e*). Interestingly, this significant disruption of proper temporal activation of chromosome condensation does not affect the cell cycle progression. The proportion of cells in different stages of the cell cycle was similar in both parental *Mcph1*+/+ and Δ*Mcph1* cell lines, which is consistent with previous findings (Arroyo et al., 2017; Houlard et al., 2021) (Fig. 2*f*).

Additionally we measured the metaphase chromosome length from Δ*Mcph1* cell lines and compared it to the parental line. Our analyses demonstrate that metaphase chromosomes in Mcph1-depleted cells are significantly shorter than the chromosomes of the parental line (Fig. 2*g*, *h*).

Moreover, we observed a significant increase in micronuclei in Mcph1-lacking cells (Supplementary Material 3). In two out of three Δ*Mcph1* cell lines we detected the formation of a Robertsonian metacentric chromosome by the fusion of two acrocentric chromosomes (marked by the red arrowhead at Fig. 2*g*).

Effects of Mcph1 knockout on gene expression in mESCs

To determine if specific interphase chromatin features affect gene expression, we conducted a transcriptome analysis in the Δ*Mcph1* cell lines and the control parental *Mcph1*+/+ cell line. RNA-seq also confirmed the deletion of exon 2 of the *Mcph1* gene in all targeted cell lines (Fig. 3*a*). The absence of transcripts aligning to the second exon in Δ*Mcph1* cell lines unequivocally indicates successful CRISPR/Cas9-mediated targeting. According to RNA-seq data, the expression level of *Mcph1* in knockout cell lines decreases threefold (p-value = 1.05e–15) compared to the parental cell line, likely due to the activation of the nonsense-mediated RNA decay mechanism (Brogna, Wen, 2009).

To determine the changes in gene expression following *Mcph1* knockout, we analyzed RNA-seq data from three independently derived knockout cell lines and compared them with three replicates of the parental cell line. Genes with a base mean expression < 100 were excluded from analysis. We found that 876 genes significantly changed their expression level (twofold or more) after *Mcph1* knockout (see Supplementary Material 1 for the whole list of differentially expressed genes (DEGs)). These DEGs are equally distributed between up- and downregulated genes' groups. Classification by Gene Onto-

[1] Supplementary Materials 1–3 are available at:
https://vavilovj-icg.ru/download/pict-2024-28/appx18.xlsx

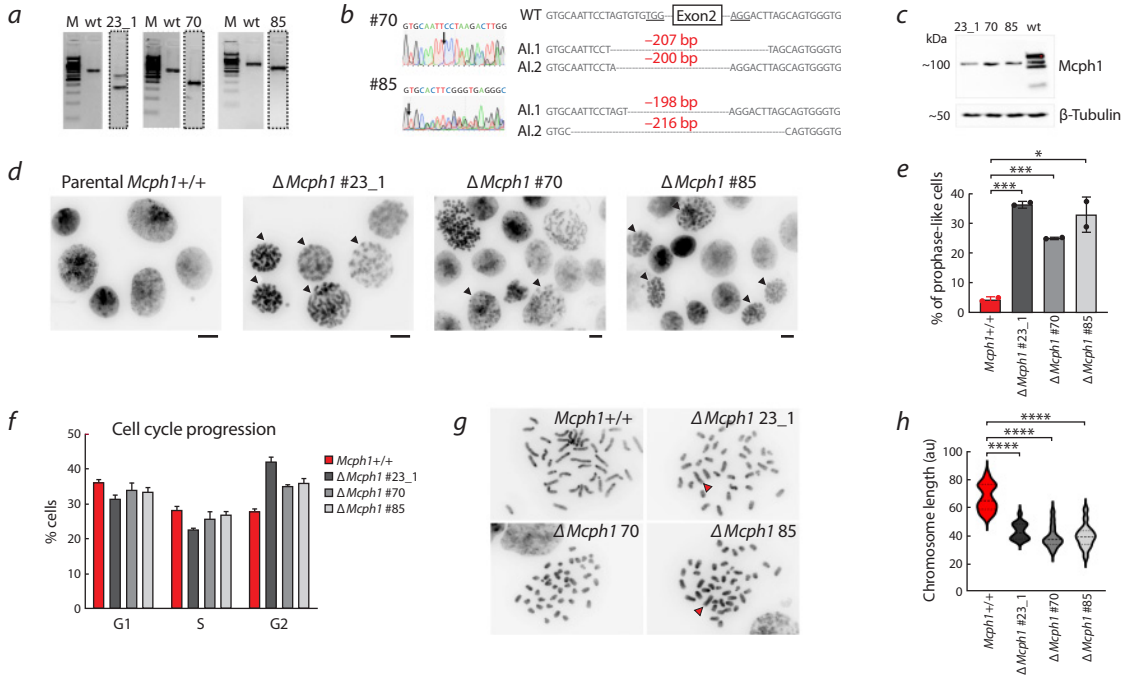

Fig. 2. The deletion of *Mcph1* in mESCs induces chromosome condensation and metaphase chromosome shortening.

a, Representative PCR genotyping of genome-edited mESCs clones (three potential clones are shown as an example). *b*, Genotyping of the potential clones by Sanger sequencing. *c*, Western blot analysis of parental *Mcph1*+/+ and Δ*Mcph1* cell lines. *d*, Representative images of prophase-like nuclei (arrowheads) observed in the Δ*Mcph1* cell lines. The nuclei were visualized through DAPI staining. Scale bar: 10 μm. *e*, Quantification of the percentage of prophase-like nuclei cells. Data represent the mean of two independent experiments ± SD. A minimum of 134 cells was examined in each experiment. Two-sided Student's t test. *f*, Cell-cycle analysis through propidium iodide flow cytometry in parental *Mcph1*+/+ and Δ*Mcph1* cell lines. *g*, Representative images from a normal-sized metaphase and a metaphase with hypercondensed chromosomes in Δ*Mcph1* cell lines. *h*, Mean length of all chromosomes in parental *Mcph1*+/+ and Δ*Mcph1* cell lines. The lengths were measured in arbitrary units (au); 15 metaphases were examined for each sample. One-way ANOVA followed by Dunnett's test.

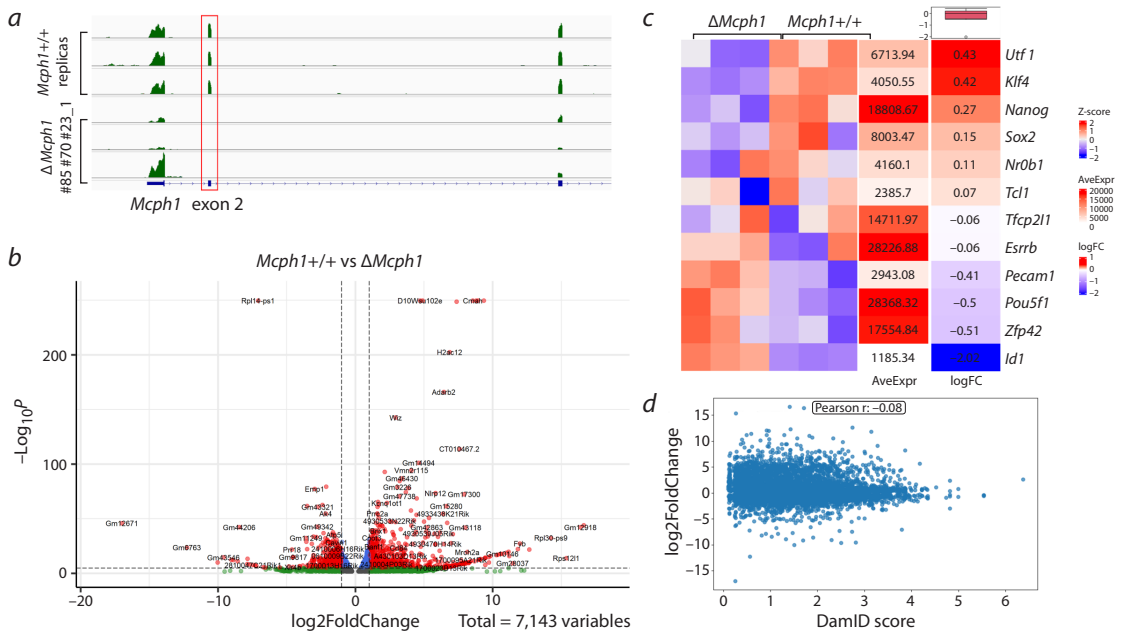

Fig. 3. Effects of Mcph1 depletion on gene expression in mESCs.

a, RNA sequencing coverage across the first three exons of *Mcph1* in Δ*Mcph1* cell lines. *b*, Volcano plot of the significant DEGs between parental cell lines and Δ*Mcph1* cell lines. The x-axis represents the log2 fold change and the y-axis represents –log10 of each significant DEG. Red spots beyond the dashed lines are considered to be significantly expressed at $p \leq 0.05$. *c*, Heat map of pluripotency gene expression values for all the cell lines used. Each horizontal line represents a gene and each column represents a single sample. The color intensity reflects the level of gene expression (red for upregulation and blue for downregulation). *d*, The correlation between DEGs and LADs. For the gene sets with significantly altered gene expression after *Mcph1* knockout (log2 fold change y-axis) DamID contact frequency scores are shown (x-axis).

Gene Ontology categories with FDR < 0.05 enriched after *Mcph1* knockout in mESCs

GO biological process complete	Over/under	Fold enrichment	Raw *p*-value	FDR
Sensory perception of smell (GO:0007608)	+	7.48	3.68E–08	4.95E–04
Sensory perception of chemical stimulus (GO:0007606)	+	6.75	4.04E–08	2.72E–04
Oxidative phosphorylation (GO:0006119)	+	3.39	5.83E–06	1.57E–02
Organic substance metabolic process (GO:0071704)	–	0.81	5.66E–06	1.90E–02
Primary metabolic process (GO:0044238)	–	0.80	6.55E–06	1.47E–02
Nitrogen compound metabolic process (GO:0006807)	–	0.79	8.59E–06	1.65E–02
Macromolecule metabolic process (GO:0043170)	–	0.76	2.14E–06	9.60E–03

logy (GO) terms revealed 5 significantly-affected categories (FDR *p*-value < 0.05) related to general metabolism and olfactory receptor activity (see the Table). Interestingly, terms of sensory perception were not attributed to *Mcph1* knockout before. While oxidative phosphorylation was highlighted as one of the most affected pathways in primary cultures of neural progenitors from *Mcph1* full-knockout mice (Journiac et al., 2020).

We did not observe an enrichment of regulated genes associated with cell cycle control – pathways in which Mcph1 is known to be involved (Yang et al., 2008). In detail, there were no significant differences in the expression levels of *Chk1*, *Brca1*, *Topbp1*, *Ddb2*, *p73* and *Tert*, which were all reported to show reduced expression following *Mcph1* knockout (Yang et al., 2008). Contrary to previous reports, we observed a slight but significant upregulation of *Rad51* and *Apaf1* expression level in Δ*Mcph1* cell lines (log2FoldChange = –0.81, adjusted *p*-value =3.27×10^{-9} for *Rad51*; log2FoldChange = –0.6, adjusted *p*-value = 6.38×10^{-5} for *Apaf1*).

One of the hallmarks of embryonic stem cells is their ability to differentiate into almost any cell type. Thus, the high number of differentially expressed genes between parental *Mcph1*+/+ and Δ*Mcph1* cell lines might be a consequence of cell differentiation after Mcph1 depletion. To test this hypothesis, we have further analyzed the expression levels of key pluripotency markers such as *Sox2*, *Pou5f1*, *Nanog*, *Klf4*, etc. We have not observed any significant or consistent decrease in expression of these genes thereby indicating that differentiation had not taken place (Fig. 3*c*). Thus, Mcph1 is involved in the regulation of pluripotency in mESCs neither directly nor indirectly through influencing global chromatin organization.

The Mcph1 depletion induces significant remodeling of nuclear chromatin due to chromosome condensation. It can be hypothesized that the formation of rod-shaped chromosomes during interphase may cause disruptions in chromatin association with the nuclear lamina. Thus, we decided to find a correlation between alterations in gene expression level and frequency of contact with the lamina. Lamina-associated domains (LADs) regions in mESCs were identified by DamID-seq of Lamin B1 (Borsos et al., 2019). We found no correlation between the DamID contact frequency scores and changes in gene expression in mutant Mcph1 cells (Fig. 3*d*). These data suggest that Mcph1-mediated premature chromosome condensation during interphase is not the one that leads to changes in gene expression patterns of mESCs.

Discussion

Microcephalin (Mcph1) is found in all metazoa. This multifaceted protein plays an important role in multiple fundamental cellular processes including DNA damage repair, cell-cycle progression and apoptosis, regulation of chromosome condensation and centrosome biogenesis. Loss-of-function mutations of *Mcph1* cause primary microcephaly, associated with severe reduction in brain volume and clinical decline in neurocognitive function (Jackson et al., 2002). Previous studies have shown that the expression level of *Mcph1* is decreased in many types of cancers including breast cancer, lung cancer, cervical cancer, etc. compared to normal tissue (Alsolami et al., 2023). Thus, *Mcph1* has attracted intense research interest due to its crucial role in neurogenesis and cancer suppression (Pulvers et al., 2015; Liu et al., 2016).

Numerous studies have implied that Mcph1 plays an important role in chromosome maintenance (Arroyo et al., 2017; Cicconi et al., 2020). Tracking the dynamics of mitosis progression in Mcph1-depleted cells in real time revealed a range of anaphase defects and missegregated chromosomes that become encapsulated in micronuclei (Arroyo et al., 2017). Mcph1 specifically interacts with TRF2 in the shelterin complex of telomeric DNA and promotes homology-directed repair of dysfunctional telomeres. Moreover, Mcph1 supports telomere replication during the S-phase of the cell cycle by counteracting replication stress (Cicconi et al., 2020). In our study we also observed an elevated frequency of chromosomal abnormalities including micronuclei and Robertsonian translocations in the knockout Δ*Mcph1* lines (Supplementary Material 3). According to the previously published data we also detected a significant reduction in chromosome length for all Δ*Mcph1* cell lines (Gruber et al., 2011; Arroyo et al., 2017) (Fig. 2*h*). A similar phenomenon of hypercondensed metaphase chromosomes was also observed in cells continuously treated with nocodazole resulting in spindle destruction and significant prolonged mitosis (Naumova et al., 2013). Thus, increasing the duration of condensin loading to chromatin either by prolonged metaphase arrest after nocodazole treatment or chromosome condensation in interphase nuclei mediated by *Mcph1* knockout leads to the shortening of mitotic chromosomes.

Several studies reported the transcriptional activity of MCPH1 (Lin, Elledge, 2003; Yang et al., 2008; Shi et al., 2012). It was shown that in HEK293 cells MCPH1 acts as a coactivator by forming a complex with the transcription factor E2F1 and regulates a number of genes (such as *CHK1*

and *BRCA1*) involved in DNA repair, the cell cycle and apoptosis (Yang et al., 2008). Furthermore, *MCPH1* was first identified as an inhibitor of hTERT expression – that is why *MCPH1* is also called *BRIT1* (BRCT-repeat inhibitor of TERT expression) (Lin, Elledge, 2003). Later it was demonstrated that MCPH1 directly binds to the hTERT proximal promoter leading to reduced hTERT expression and telomerase activity (Shi et al., 2012). Comparative gene expression profiling of neural progenitors in *Mcph1* knockout and wild-type mice has revealed altered expression of genes controlling the cell cycle and genes related to metabolic pathways (Journiac et al., 2020). In our study we investigated the changes in the transcriptional profiles of mESCs after *Mcph1* knockout. Among significantly upregulated and downregulated (876) DEGs, GO analysis revealed enrichment for general metabolism and sensory perception of smell. Although it is hard to draw direct connections to the known Mcph1 functions, these data show that mESCs may try to adapt their metabolism to chronic chromatin hypercondensation. Furthermore, contrary to the aforementioned studies, we found no significant differences in the expression levels for *Tert* or for genes implicated in the cell cycle pathway after *Mcph1* knockout in mESCs. Thus, contribution of Mcph1 to the regulation of gene expression appears to be species- and tissue-specific. This is also supported by the fact that most of the human-specific amino acid substitutions in MCPH1 resulted in changes in the regulatory effects on the downstream genes (Shi et al., 2013).

It is now established that spatial organization of chromatin in the nucleus is important for proper regulation of gene expression. *Mcph1* knockout results in the loading of condensin II onto chromatin followed by chromosome condensation during interphase. It is possible to assume that at least a part of the expression changes after *Mcph1* knockout could be explained by alterations in chromatin spatial organization. It was previously shown that condensin II depletion contributes to the folding of the human genome by shifting from chromosome territories to Rabl-like polarized organization with chromocenter formation (Hoencamp et al., 2021). Such drastic reorganization affects the expression of a small fraction of genes within LADs and near LAD borders (Hoencamp et al., 2021). Knockout of *Mcph1* also leads to large-scale reorganization but in the opposite manner: interphase chromosomes are individualized into prophase-like rod-shaped chromatids, while chromocenters have disappeared. In our transcriptome analysis of mESCs with *Mcph1* knockout, we found no correlation between changes in the expression level of genes and their proximity to lamina. Thus, loading of condensin II onto chromatin does not affects smaller-scale chromatin structures such as LADs and TADs (topology associated domains) contributing to the regulation of gene expression.

Conclusion

In this work we have generated mESCs with a knockout of the *Mcph1* gene. Our conclusion is that Mcph1 is likely not involved in the regulation of gene expression in mESCs by direct binding to target promoters or by modulation of spatial chromatin organization, while the DEGs observed may be the result of secondary effects due to persistent chromatin hypercondensation. These cell lines will be a valuable resource for investigating Mcph1-condensin II pathway in chromosome maintenance, and could also be used to study Mcph1 roles in DNA repair.

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A comparative study on germination of wheat grains with different anthocyanin pigmentation of the pericarp in natural or induced aging

E.I. Gordeeva [1] ✉, O.Y. Shoeva [1], E.K. Khlestkina [1, 2]

[1] Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
[2] N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), St. Petersburg, Russia
✉ elgordeeva@bionet.nsc.ru

Abstract. One of promising areas of wheat breeding is the creation of varieties with a high concentration of anthocyanins in the grain for the production of functional food products. Nonetheless, the question of how these compounds affect seed viability after long-term storage has remained unexplored. A comparative study on seed viability was conducted using a set of near-isogenic lines on the background of spring wheat variety Saratovskaya 29. These sister lines carry different combinations of recombinant DNA regions (on chromosomes 2A and 7D) containing dominant and recessive alleles at loci *Pp3* and *Pp-D1* (*Pp: Purple pericarp*), which determine the anthocyanin color of coleoptiles and of the pericarp. Seeds were germinated on two layers of water-moistened filter paper in a climatic chamber at a constant temperature of 20 °C on a 12-hour daylight cycle. During long-term natural storage of the seeds for up to 9 years in a dry ventilated room in Kraft bags at 20 ± 2 °C, the tested wheat samples experienced a loss of seed germination capacity of ~50 %; anthocyanins were found to not participate in the preservation of germination capacity. Nonetheless, anthocyanins contributed to the preservation of seed viability under unfavorable short-term conditions of a temperature rise to 48 °C at 100 % humidity. The accelerated aging test did not predict poor germination capacity after long-term seed storage. The results showed a neutral role of anthocyanins in the maintenance of seed germination capacity for 6–9 years under natural storage conditions at 20 ± 2 °C. A small statistically significant increase in grain germination capacity during natural aging was associated with the presence of a recombinant region containing the *Pp-D1* gene on wheat chromosome 7D.

Key words: wheat; anthocyanin; natural aging; seed germination.

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Сравнительное изучение прорастания семян пшеницы, различающихся антоциановой окраской перикарпа, в условиях естественного и индуцированного старения

Е.И. Гордеева [1] ✉, О.Ю. Шоева [1], Е.К. Хлесткина [1, 2]

[1] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Федеральный исследовательский центр Всероссийский институт генетических ресурсов растений им. Н.И. Вавилова (ВИР), Санкт-Петербург, Россия
✉ elgordeeva@bionet.nsc.ru

Аннотация. Одним из перспективных направлений селекции пшеницы является получение сортов с повышенным содержанием антоцианов в зерновке для производства функциональных продуктов питания. Однако вопрос о том, как эти соединения влияют на жизнеспособность семян после длительного хранения, оставался неизученным. Сравнительное исследование жизнеспособности семян было проведено с использованием набора почти изогенных линий пшеницы сорта Саратовская 29. Эти сестринские линии имеют различные сочетания рекомбинантных участков ДНК в хромосомах 2A и 7D с доминантными и рецессивными аллелями генов *Pp3* и *Pp-D1* (*Pp*, *Purple pericarp*), контролирующих антоциановую окраску колеоптилей и околоплодника. Семена про-

ращивали в чашках Петри на увлажненной фильтровальной бумаге в климатической камере при постоянной температуре 20 °C с 12-часовым циклом дневного освещения. При длительном естественном хранении семян до 9 лет в сухом проветриваемом помещении в крафт-пакетах при температуре 20 ± 2 °C у испытанных образцов пшеницы происходила потеря всхожести семян до 50 %. При этом положительного влияния наличия антоцианов в зерне на сохранение всхожести не выявлено. Однако антоцианы способствовали сохранению жизнеспособности зерен в неблагоприятных кратковременных условиях повышения температуры до 48 °C и 100 % влажности. Тест на индуцированное старение не позволил предсказать ухудшение прорастания после длительного хранения семян. Результаты исследования показали нейтральную роль антоцианов в сохранении прорастания семян в течение 6–9 лет в естественных условиях хранения при 20 ± 2 °C. Небольшое статистически достоверное повышение всхожести зерен при естественном старении было связано с наличием рекомбинантного участка в хромосоме 7D пшеницы, содержащего ген *Pp-D1*.

Ключевые слова: пшеница; антоцианы; естественное старение; жизнеспособность семян.

Introduction

Bread wheat is one of the most important grain crops ensuring this country's food security. Currently, there is increasing interest in the growing of wheat with a high concentration of anthocyanins in grain bran. It is not only a resource of stress resistance and plant adaptability (Kaur et al., 2023), but also a source of functional foods (beneficial to human health) and a possible therapeutic agent (Yudina et al., 2021; Liu et al., 2021; Loskutov, Khlestkina, 2021; Garg et al., 2022).

Anthocyanins are plant pigments belonging to the class of flavonoid compounds (Patra et al., 2022). They take part in the protection of plants from excess ultraviolet radiation and from pathogens and play the role of attractants for insects and animals for pollination of flowers and for seed dispersal (Corso et al., 2020). As biologically active secondary metabolites with antioxidant properties, these compounds can neutralize cell-damaging reactive oxygen species (ROS) that accumulate during normal metabolism or stress (Shen et al., 2022). Despite the advent of wheat varieties that accumulate anthocyanin pigments in the caryopsis, the relation between the biosynthesis of these compounds and their protective and adaptive ecological functions remains unexplored, as do mechanisms maintaining seed viability, that is, the ability to produce normal seedlings under favorable conditions after long-term storage.

Wheat – just as most angiosperms common in regions with a temperate climate and large seasonal temperature fluctuations – has orthodox, desiccation-tolerant, ripened seeds. Their moisture content drops below 10 %, which reduces cellular activity (mobility of molecules) inside the seeds to a minimal level and allows to maintain viable dormant embryos in a state of anabiosis for a long period (Guryeva et al., 2021). This state of minimal cellular activity represents a highly successful strategy for plants to survive under adverse environmental conditions, thereby extending their longevity.

Seed longevity is a polygenic trait and is regulated by a complex interaction of variable environmental factors (such as temperature, relative humidity, and partial pressure of oxygen) with endogenous genetically controlled factors of plants. The latter factors include seed coat structure, the concentration of ROS, the integrity of phospholipid layers, proteins, nucleic acids (and associated repair systems), energy reserves (sugars) in the endosperm, and a balance of dormancy phytohormones and seed germination (Zhou W. et al., 2020).

Molecular mechanisms underlying the processes of seed viability and longevity are currently being actively studied (Li et al., 2022; Stegner et al., 2022). It is known that the dormant stage of seeds is controlled by a phytohormone called abscisic acid, and on the contrary, phytohormones gibberellins participate in seed germination: they are antagonists of abscisic acid (Longo et al., 2020). Plant hormones, together with ROS (such as the superoxide anion, hydrogen peroxide, and hydroxyl and peroxyl radicals), are components of the regulatory signaling system responsible for the sensing of (and adaptation of plant metabolism to) stress and participate in the control of developmental and growth processes as well as in protection from pathogens (Kurek et al., 2019; Considine, Foyer, 2021). For example, hydrogen peroxide causes the catabolism of abscisic acid and stimulates the biosynthesis of gibberellins, thereby promoting exit from dormancy and triggering seed germination (Chen et al., 2018). Regulation of ROS accumulation should be under strict control of antioxidants. When the balance between pro- and antioxidant processes is disturbed, oxidative stress takes place, causing protein modifications, lipid peroxidation, membrane damage (with elevated leakage of electrolytes and mitochondrial degradation), and lesions in DNA and RNA; these events lead to cell death and ultimately a loss of seed viability (Kurek et al., 2019; Li et al., 2022).

To ensure homeostasis and diminish excessive levels of ROS, plants activate internal defense systems, such as enzymatic and nonenzymatic antioxidants (Kumar et al., 2020). Enzymatic antioxidants include superoxide dismutase, catalase, and enzymes of the glutathione-ascorbate cycle, the activity of which sharply decreases in dry seeds owing to cytoplasm viscosity. The nonenzymatic antioxidant system is represented by molecules of ascorbic acid, glutathione, lipophilic tocopherols (vitamin E), carotenoids, and a large class of phenolic compounds (Dogra, Kim, 2020; Kumar et al., 2020; Dumanović et al., 2021).

Seed viability is closely related to the morphological structure of the seed coat and to the concentration of phenolic compounds in it (Sano et al., 2016). The seed coat plays the part of a physical barrier to external adverse factors by limiting water absorption and damage by fungi and microbes (Rathod et al., 2017; Zhou W. et al., 2020). As demonstrated in mutant *Arabidopsis thaliana* plants, defects in flavonoid pigmentation reduce the permeability of the seed coat and as a consequence affect seed survival (Sano et al., 2016). For instance, in a study on mutants *tt2*, *tt10*, and *tt12*, a connection was found between a decrease in the concentration of pigments called proanthocyanidins (polymeric flavonoids located in the endothelium of the seed coat and in chalaza cells) and a shortening of seed

lifespan (Debeaujon et al., 2001). The *tt10* mutants have a phenotype of delayed seed coat browning, which is associated with the formation of condensed tannins by the product of the *TRANSPARENT TESTA 10* (*TT10*) gene encoding laccase-like 15-flavonoid oxidase (AtLAC15), and a concomitant reduction in seed dormancy and lifespan (Pourcel et al., 2007).

Biosynthesis of flavonols and proanthocyanidins (which are precursors of highly polymerized insoluble pigments) in the seed coat of the red-grained wheat caryopsis is associated with greater dormancy and resistance to germination before harvest as compared to white-grained forms (Kohyama et al., 2017; Mares, Himi, 2021). Polyphenols are positively connected with the control of seed dormancy owing to their influence on the transcription of genes related to the production of phytohormones (abscisic, salicylic, and jasmonic acids; gibberellins; and polyethylene) as well as to the removal of ROS (Shah et al., 2018; Zhou G. et al., 2023). It has been shown that water-soluble phenolic compounds in the wheat caryopsis coat act as endogenous inhibitors on germination processes and partially inhibit peroxidase activation (Kong et al., 2008).

At increased temperature of storage and high humidity, the oxidation of fats and proteins and disturbances of nucleic-acid integrity are accelerated, whereas seed longevity is markedly reduced (Zhou W. et al., 2020). In this way, it is possible to emulate natural aging of seeds. This phenomenon has been used to develop the "accelerated aging test" (AA test) (Rehman Arif et al., 2012; Hay et al., 2019). Tests of germination vigor and seed viability have been validated and included in the International Seed Testing Association's (ISTA) seed testing guidelines (International Rules…, 2004).

The purpose of the present work was a comparative study on seed viability of wheat near-isogenic lines (NILs) featuring the presence of recombinant regions (on chromosomes 2A and 7D) carrying *Pp* (*Purple pericarp*) genes (which regulate the biosynthesis of anthocyanins in the caryopsis pericarp) after natural long-term storage and artificially induced aging of the seeds. The obtained data will allow to answer the question whether the accumulation of anthocyanins – which have antioxidant properties – in the wheat caryopsis pericarp affects seed longevity.

Materials and methods

Plant material. Seed germination capacity was assessed in seven sister lines (NILs) of wheat that were created from a spring variety of common wheat – Saratovskaya 29 (S29) – via crosses with donors of dominant alleles of *Pp* genes [varieties Purple (P) and Purple Feed (PF)] and selection of purple-grained hybrid plants in $BC_{8\text{-}9}F_2$ (Arbuzova et al., 1998; Gordeeva et al., 2015). These lines are characterized by the presence (in chromosomes 2A and 7D) of recombinant DNA regions inherited from the donor lines and containing genes *Pp3* and *Pp-D1* (Tereshchenko et al., 2012; Gordeeva et al., 2015). A brief description of the lines is given in Table 1 and Figure 1.

When conditions for accelerated induced aging (AA test) were being chosen, seeds of red-grained winter variety Mironovskaya 808, of white-grained spring variety Novosibirskaya 67, and of red-grained spring varieties Saratovskaya 29 and Chinese Spring were used, from the GenAgro collection [Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (ICG SB RAS), Novosibirsk, Russia].

The method of accelerated seed aging. For induced seed aging, the AA test developed by the ISTA was employed, with modifications. Seeds from varieties Mironovskaya 808, Novosibirskaya 67, Saratovskaya 29, and Chinese Spring – grown under identical conditions of one growing season in a hydroponic greenhouse – were used to find temperature conditions for the AA test.

Fifty seeds of each genotype in triplicate were placed on stainless-steel meshes set above distilled water in plastic cups covered with waterproof film. The cups were kept either at an

Table 1. Wheat samples used in this study

Line ID #	Cultivar / Line genetical name	Short name	Short description
1	cv. Saratovskaya 29 S29*pp3pp-D1*	S29	Red-grained spring variety
2	i:S29*Pp3*^{P}*pp-D1*	S29*Pp3*^{P}	Red-grained isogenic line S29 with a recombinant region (on chromosome 2A) containing a dominant allele of the *Pp3* gene from variety Purple
3	i:S29*pp3Pp-D1*^{P}	S29*Pp-D1*^{P}	Red-grained isogenic line S29 with a recombinant region (on chromosome 7D) containing a dominant allele of the *Pp-D1* gene from variety Purple
4	i:S29*Pp3*^{P}*Pp-D1*^{P}	S29*Pp3Pp-D1*^{P}	Purple-grained isogenic line S29 with two recombinant regions (on chromosomes 2A and 7D) containing dominant alleles of genes *Pp3* and *Pp-D1* from variety Purple
5	i:S29*Pp3*^{PF}*pp-D1*	S29*Pp3*^{PF}	Red-grained isogenic line S29 with a recombinant region (on chromosome 2A) containing a dominant allele of the *Pp3* gene from variety Purple Feed
6	i:S29*pp3Pp-D1*^{PF}	S29*Pp-D1*^{PF}	Red-grained isogenic line S29 with a recombinant region (on chromosome 7D) containing a dominant allele of the *Pp-D1* gene from variety Purple Feed
7	i:S29*Pp3*^{PF}*Pp-D1*^{PF}	S29*Pp3Pp-D1*^{PF}	Purple-grained isogenic line S29 with two recombinant regions (on chromosomes 2A and 7D) containing dominant alleles of the *Pp* genes from variety Purple Feed

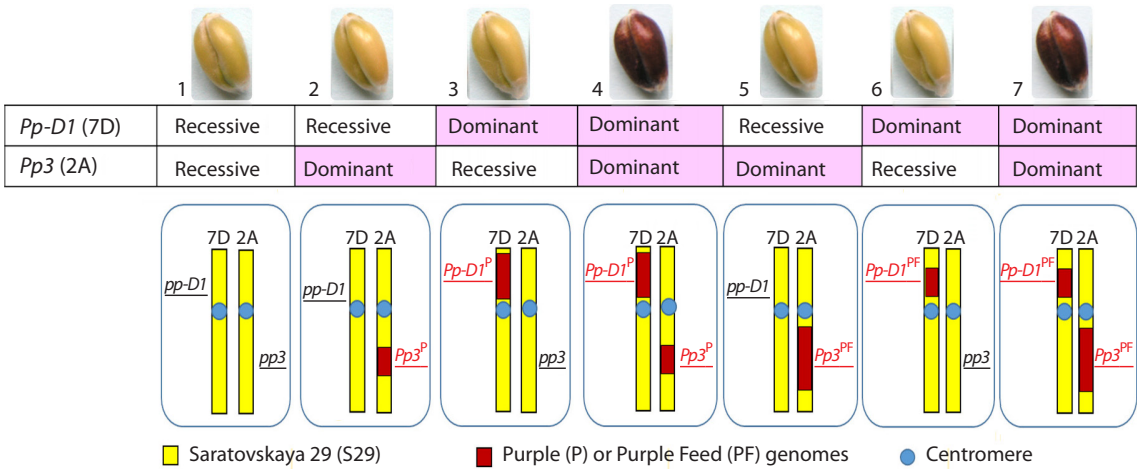

Fig. 1. The grains and schematic representation of chromosomes 2A and 7D carrying recombinant regions containing anthocyanin biosynthesis–regulatory genes in the wheat NILs used in the natural aging tests.

elevated temperature (42, 44, 46, or 48 °C) or at 20 °C (control) with 100 % humidity for 72 h in a Rubarth Apparate climatic chamber (RUMED GmbH, Germany). The seeds were then transferred to 24 24 cm Petri dishes onto two-layer moist filter paper and placed in the climatic chamber at 20 °C with 12-h lighting for germination. The vigor of seed germination as a percentage was determined as the ratio of the number of seeds that germinated within 72 h (on the third day) to the total number of analyzed seeds in triplicate. Seed viability (%) was determined as the number of seeds that germinated after seven days to the total number of analyzed seeds in triplicate. Only healthy green seedlings with a normal root system without anomalies were included in the calculations [GOST (Russian quality standard) No. 12038-84] (Fig. 2).

The germination index after artificial (induced) aging was calculated by means of the formula:

$$\text{Germination index (\%)} = \frac{\text{Normal germinated grains after 48 °C treatment and 7 days of germination}}{\text{Normal germinated grains in control, i. e., after 20 °C treatment and 7 days of germination}} \times 100\ \%.$$

Based on the assessment results, a temperature was chosen for the AA test of the studied NILs of the Saratovskaya 29 variety. Seeds of these lines were collected either after the spring growing season of 2012 in a greenhouse or on an experimental plot at a selection/genetic center at the ICG SB RAS in 2012. Before the experiment, the seeds were stored for 2 months in Kraft bags at 20 ± 2 °C. The AA test was performed similarly to the experiment with the selection of temperature conditions, except that instead of fifty, one-hundred seeds of each genotype were used. Significance of differences between parent variety Saratovskaya 29 and sister NILs was evaluated as three biological replicates by the Mann–Whitney U test; at $p < 0.05$, differences were considered significant.

Natural aging of grains. To test seed germination capacity under natural aging conditions, seeds of the analyzed lines were collected from plants grown in the greenhouse of the ICG SB RAS from 2014 to 2017 and in 2021 (for control). The seeds were stored in Kraft paper bags at 20 ± 2 °C, and their germination capacity was assessed in 2023 after 6–9 years of storage. Seeds after two years of storage served as a control.

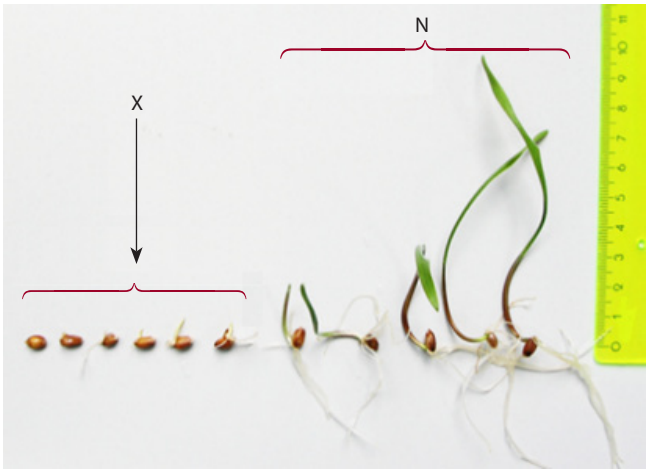

Fig. 2. Seedlings' performance after a standard germination test.
X = abnormal seedlings; N = normal germination.

One-hundred seeds of each NIL were germinated in triplicate in 24 × 24 cm Petri dishes on two layers of moistened filter paper. The Petri dishes were placed in the Rubarth Apparate climatic chamber, incubated for 24 h at 4 °C in the dark to synchronize germination, and then were germinated at a constant temperature of 20 °C on a 12-h/12-h light/dark cycle. Germination vigor and seed viability were determined at days three and seven, respectively, after the germination initiation. Seed germination vigor as a percentage was determined as the ratio of the number of seeds that germinated within 72 h (on the third day) to the total number of analyzed seeds in triplicate. Seed viability (%) was determined as the number of seeds that germinated after seven days to the total number of analyzed seeds in triplicate. The significance of differences between parent variety Saratovskaya 29 and sister NILs was evaluated as three biological replicates by the Mann–Whitney test (U test); at $p < 0.05$, differences were considered significant.

Results

Seed germination after induced aging

To find conditions for the AA test, germination capacity was tested in four varieties of bread wheat after heat treatment of seeds at 42, 44, 46, or 48 °C with high air humidity for 72 h. The results are presented in Table 2. Varieties Saratovskaya 29 and Chinese Spring maintained 100 % seed viability when the temperature was increased up to 46 °C, while at the same temperature, seed viability of varieties Mironovskaya 808 and Novosibirskaya 69 decreased to 78 % and 96 %, respectively. With a further increase in temperature by two degrees, all varieties manifested a decrease in seed viability. Seed viability of the red-grained winter variety Mironovskaya 808 was 52 %: inferior to that of the white-grained spring variety Novosibirskaya 67 showing a seed viability of 64 %. Seed viability of red-grained spring varieties Saratovskaya 29 and Chinese Spring after such heat treatment was 87 and 86 %, respectively. Since it was after 48 °C heat treatment that all varieties showed a decrease in seed viability and differences in this parameter, further comparative analysis of germination – by the AA test in the NILs featuring the presence of anthocyanin pigmentation in the grain – was carried out at this temperature.

Results of the AA test performed on the Saratovskaya 29 variety and two NILs with anthocyanin pigments in the pericarp (S29*Pp3Pp-D1*^{P} and S29*Pp3Pp-D1*^{PF}) are presented in Table 3. After artificial aging, the germination capacity of grains of the Saratovskaya 29 variety fell by 19 %, while in purple-grained lines, this parameter declined only by 4 %. Germination indices of seeds from the wheat NILs were 1.2 times higher than the germination index of Saratovskaya 29 seeds, which are not colored by anthocyanins.

At the same time, the germination capacity of grains collected from plants of these wheat lines grown in the field was also tested. After the AA test, the viability of the field grains was two times lower compared to seeds of the greenhouse origin. For instance, germination vigor of seeds of the Saratovskaya 29 variety was only ~20 % and seed viability was 35 %, whereas these parameters in grains of the S29*Pp3Pp-D1*^{P} line, which has an anthocyanin-containing pericarp, were 36 and 42 %, respectively. Thus, despite the spoilage of seeds by soil microorganisms, these results indicate resistance of anthocyanin-pigmented bread-wheat grains to elevated temperatures and high air humidity.

Seed germination after long-term natural storage

The experimental data showed that all the tested wheat samples germinated with a vengeance after two years of storage at 20±2 °C under favorable conditions in a dry ventilated room; seed germination capacity was 100 % (Tables 4 and 5).

The vigor of seed germination decreased to 30–39 % after six years and to 21–28 % after nine years of long-term natural

Table 2. The germination of wheat grains in the AA test after sowing

Varieties	Type of vegetation	Storage time of grains	Viability of seeds after 7 days of germination, %				
			Control	With heat treatment at 100 % humidity for 72 h			
			20 °C	42 °C	44 °C	46 °C	48 °C
Saratovskaya 29 (S29)	spring red-grained	2 years	100±0	100±0	100±0	100±0^{b}	87±1^{b}
Novosibirskaya 67	spring white-grained	2 years	100±0	99±1	99±1	96±4^{b}	64±2^{a}
Chinese Spring	spring red-grained	2 years	100±0	100±0	100±0	100±0^{b}	86±2^{b}
Mironovskaya 808	winter red-grained	2 years	100±0	100±0	100±0	78±7^{a}	52±13^{a}

$^{a, b}$ Different letters within a column denote statistically significant differences between lines at $p < 0.05$ (U test).

Table 3. Germination vigor (after 3 days, 72 h) and viability (after 7 days) of wheat seeds

Line ID #	Varieties or lines	Germination vigor, %, after 3 days	Seed viability, %, after 7 days	Germination index, %*
1	S29 / 20 °C	94±6	100±0	
	S29 / 48 °C	41±9^{a}	81±8^{a}	80.7
4	S29 *Pp3Pp-D1*^{P}/ 20 °C	100±0	99±1	
	S29 *Pp3Pp-D1*^{P}/ 48 °C	69±5^{b}	96±3^{b}	97.6
7	S29 *Pp3Pp-D1*^{PF}/ 20 °C	96±1	98±1	
	S29 *Pp3Pp-D1*^{PF}/ 48 °C	70±13^{b}	96±2^{b}	98.3

* The percentage of viable grains (48 °C) relative to the control (20 °C).
$^{a, b}$ Different letters in a column denote statistically significant differences between lines at $p < 0.05$ (U test).

storage (Table 4). In a comparison of germination vigor between the NILs and the parent variety Saratovskaya 29 (# 1), seeds of the line S29*Pp-D1*^{P} (# 5) with a recombinant DNA region in chromosome 2A from variety Purple Feed showed significant decrease in this indicator after 6 years, 7 years, and 8 years and 10 months of storage (Table 4).

The grains of line *S29Pp-D1*^{P} (# 3), carrying a recombinant DNA fragment from the variety Purple in chromosome 7D, had the highest germination vigor after seven years of storage. The grain germination vigor of line *S29Pp-D1*^{PF} (# 6) with a recombinant fragment in chromosome 7D was significantly exceeded in this indicator of variety Saratovskaya 29 seeds (line # 1) after 8 years and 10 months. No significant differences were found between the lines in grain germination vigor after 9 years and 2 months.

The poorest seed viability seven days after sowing of wheat grains stored for eight years and ten months was shown by line S29*Pp3*^{PF} (line # 5), and after 9 years and 2 months of storage, by line S29*Pp3*^{P} (line # 2); they carry recombinant regions (on chromosome 2A) from variety Purple Feed and variety Purple, respectively (Table 5).

The viability of purple-grained lines S29*Pp3Pp-D1*^{P} (# 4) and S29*Pp3Pp-D1*^{PF} (# 7), carrying recombinant regions from varieties Purple Feed and Purple on chromosomes 2A and 7D, was significantly lower after 8 years and 10 months of storage (45 and 44 % versus 52 % for variety Saratovskaya 29). Then, four months later, after 9 years and 2 months of storage, the seed viability levels diminished and did not differ significantly from variety Saratovskaya 29 (Table 5).

Line S29*Pp-D1*^{P} (# 3) with a recombinant region (only on chromosome 7D) from the variety Purple had the highest germination 7 days after sowing of grains stored for 6 and 7 years at 20 ± 2 °C, comparable to control grains stored for 2 years (viability 95–100 %). The germination index of seeds after 8 years and 10 months of storage for this line and line S29*Pp-D1*^{PF} (# 6), which carries recombinant regions (on chromosome 7D) from variety Purple Feed, was significantly higher than that of the parent variety Saratovskaya 29 (line # 1) (58 versus 52 %).

After long-term storage for 9 years and 2 months at 20 ± 2 °C, average seed viability in all lines was below 50 %, not significantly different from variety Saratovskaya 29 (the p50 value in Figure 3). The dependence of seed germination on the duration of storage was found to be well described by a linear regression model (coefficients of determination R^2 were statistically significant and varied among the lines from 0.592 to 0.844). For all lines, negative dependences on storage duration of grains were documented (Table 6, Fig. 3).

The highest coefficients of determination R^2 for the dependence of germination of the analyzed seed samples on storage time were noted for lines ## 4, 5, and 6 (Table 6). The lowest coefficient of determination R^2 = 0.592 and a weak depen-

Table 4. Germination vigor (at 3 days after sowing) of wheat grains stored for 2 or 6–9 years at 20 ± 2 °C

Line ID #	Line	2 years	6 years	7 years	8 years	8 y 10 m	9 y 2 m
1	S29	100 ± 1	39 ± 3	40 ± 2	25 ± 4	28 ± 5	28 ± 7
2	S29*Pp3*^{P}	100 ± 0	37 ± 1	35 ± 5	30 ± 1	32 ± 4	26 ± 6
3	S29*Pp-D1*^{P}	100 ± 0	35 ± 6	62 ± 4*	28 ± 5	29 ± 3	22 ± 4
4	S29*Pp3Pp-D1*^{P}	100 ± 1	39 ± 2	40 ± 4	34 ± 2	25 ± 6	23 ± 2
5	S29*Pp3*^{PF}	100 ± 0	30 ± 5*	32 ± 3*	31 ± 2	21 ± 1*	28 ± 2
6	S29*Pp-D1*^{PF}	100 ± 0	36 ± 3	34 ± 6	33 ± 2	37 ± 4*	27 ± 5
7	S29*Pp3Pp-D1*^{PF}	100 ± 0	32 ± 8	39 ± 3	30 ± 3	29 ± 2	21 ± 7

* Differences are significant compared to the control at $p < 0.05$ (U test).

Table 5. Viability of wheat grains (at 7 days after sowing) stored for 2 or 6–9 years at 20 ± 2 °C

Line ID #	Line	2 years	6 years	7 years	8 years	8 y 10 m	9 y 2 m
1	S29	100 ± 1	93 ± 2	79 ± 5	52 ± 1	52 ± 2	46 ± 7
2	S29*Pp3*^{P}	100 ± 0	89 ± 3	77 ± 4	66 ± 3*	55 ± 4	40 ± 6
3	S29*Pp-D1*^{P}	100 ± 0	95 ± 3	95 ± 5*	57 ± 7	58 ± 3*	43 ± 3
4	S29*Pp3Pp-D1*^{P}	100 ± 1	83 ± 4*	76 ± 4	55 ± 3	45 ± 2*	42 ± 2
5	S29*Pp3*^{PF}	100 ± 0	85 ± 3*	66 ± 6*	57 ± 5	38 ± 1*	46 ± 6
6	S29*Pp-D1*^{PF}	100 ± 0	89 ± 4	74 ± 8	54 ± 6	58 ± 4*	48 ± 9
7	S29*Pp3Pp-D1*^{PF}	100 ± 0	86 ± 6*	83 ± 3	46 ± 2*	44 ± 1*	41 ± 1

* Differences are significant compared to the control at $p < 0.05$ (U test).

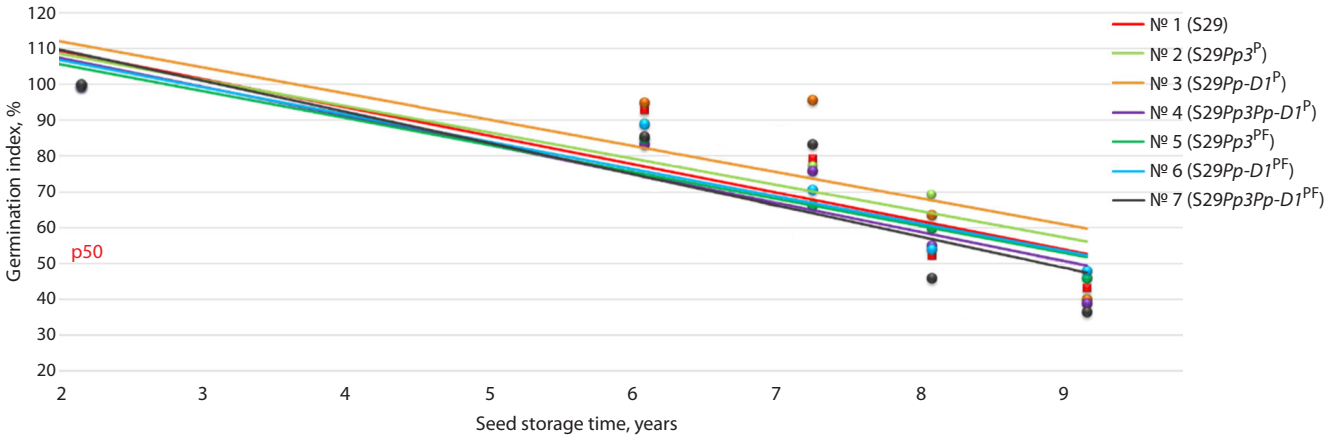

Fig. 3. The variability of grain germination after 2 and 6–9 years of seed storage at 20 ± 2 °C.

Table 6. The results of the regression analysis of grain germination variability in the wheat lines with time

line ID #	Line	Linear regression equation ($y = b_0 + b_1 \cdot x$)	R	p
1	S29	$y = 125.415 - 7.944x$	–0.876	0.0000
2	S29*Pp3*^{P}	$y = 124.454 - 7.677x$	–0.888	0.0000
3	S29*Pp-D1*^{P}	$y = 127.712 - 7.594x$	–0.769	0.0002
4	S29*Pp3Pp-D1*^{P}	$y = 125.342 - 8.527x$	–0.919	0.0000
5	S29*Pp3*^{PF}	$y = 125.718 - 8.806x$	–0.932	0.0000
6	S29*Pp-D1*^{PF}	$y = 121.357 - 7.302x$	–0.903	0.0000
7	S29*Pp3Pp-D1*^{PF}	$y = 126.469 - 8.674x$	–0.878	0.0000

dence of seed germination on storage duration was shown by line # 3, which has a single recombinant region in the short arm of chromosome pair 7D. Low coefficients of determination indicate a low quality of the constructed model, implying that seed germination is also influenced by other factors (aside from storage duration), which were not taken into account when the regression model was constructed.

In the analysis of linear regression equations, it was found that the initial germination of grains (coefficient b_0) was similar among the wheat lines. Coefficient b_1 characterizes the slope of the regression line: the higher the value of b_1, the more sensitive the lines are to the storage of grains. The highest b_1 values were registered in lines # 7 S29*Pp3Pp-D1*^{PF} (b_1 = –8.674), # 4 S29*Pp3Pp-D1*^{P} (b_1 = –8.527), and # 5 S29*Pp3*^{PF} (b_1 = –8.806), which carry recombinant regions (on chromosome 2A) from donors. By contrast, the lowest b_1 values were obtained for lines # 6 S29*Pp-D1*^{PF} (b_1 = –7.302) and # 3 S29*Pp-D1*^{P} (b_1 = –7.594), which are characterized by the presence of a recombinant region from a donor on chromosome 7D.

In contrast to the positive effect of anthocyanins on seed germination after accelerated induced aging, a role of anthocyanins in the maintenance of the viability of bread-wheat seeds under long-term storage conditions was not detectable; however, an influence of a recombinant region from chromosome 7D was noted.

Discussion

Induced seed aging and viability

It is generally accepted that at high humidity and increased storage temperature, an accelerated loss of seed viability takes place. The AA test, as a controlled spoilage procedure, emulates natural aging of seeds and allows one to assess their viability.

Grains of several spring and winter varieties of bread wheat, grown under identical controlled conditions in a greenhouse and stored for less than a year after harvesting, were tested in this work; a pre-sowing seed treatment temperature (48 °C) at high air humidity for 72 h was found for the AA test. Only after seed pretreatment temperature was raised to 48 °C, did seed viability of red-grained spring wheat varieties Saratovskaya 29 and Chinese spring diminish, to 87 and 86 %, respectively. Seed viability of the Siberian white-grained variety Novosibirskaya 67 decreased to 64 %. Of note, the lowest seed viability was recorded for grains of winter variety Mironovskaya 808: only 52 %.

According to literature data, at the Institute of Plant Genetics and Crop Research (IPK Gatersleben, Germany), a collection of winter wheat grains and synthetics has been subjected to artificial aging: kept for 72 h at 43 °C with high humidity (~100 %) (Landjeva et al., 2010; Rehman Arif et al., 2012; Agacka-Mołdoch et al., 2016; Arif et al., 2017). In contrast,

in a study on a drought-tolerant red-grained dihaploid wheat population at Shanxi Agricultural University (China), grains were kept for 0, 24, 36, 48, 60, or 72 h at a higher temperature of 48 °C (Shi et al., 2020). These data indicate that seeds of spring red-grained wheat varieties are more resistant to brief increases in temperature and humidity.

Previously, it has been reported that on the long arm of chromosome 3A, a mutation of a functional allele of the *R1* gene (*Tamyb10-A1*), which codes for a transcription factor of the R2R3-MYB type and regulates the flavonoid biosynthesis pathway, gives rise to a white shell of the wheat grain and to a decrease in the dormancy period (Mares, Himi, 2021). Those authors hypothesized that by itself the red color of the seed coat is not absolutely necessary for dormancy. It had a cumulative effect in combination with other dormancy control loci unrelated to the grain color because the exit from dormancy occurred earlier in isolated embryos than in intact hulled caryopses. Thus, the functional allele of the *R1* gene enhanced the expression of genes that control dormancy in the wheat caryopsis and extended the time of exit from dormancy (Mares, Himi, 2021).

Even though the red-grained wheat variety Saratovskaya 29 is more viable in comparison with white-grained and winter varieties, in the NILs with anthocyanin pigmentation of the grain that were derived from it, the germination index was significantly higher (by ~20 %) after artificial aging as compared with the red-grained variety Saratovskaya 29 (Table 3). Higher viability of grains of NILs having an anthocyanin-containing pericarp in comparison with the red-grained parent variety was also observed in field harvest seeds, which were infected with pathogens and fungi. This effect of anthocyanins can be explained by their antioxidant properties and participation in the neutralization of ROS arising under the conditions of elevated temperature and humidity. Thus, a positive relation between the content of anthocyanin pigments in the pericarp of spring bread wheat Saratovskaya 29 and the preservation of the viability of dormant seeds after a short increase in ambient temperature to 48 °C at 100 % air humidity was demonstrated. This phenomenon can be explained by the action of *Pp* genes' products triggering the biosynthesis of anthocyanins (which have antioxidant potential) in the pericarp of wheat grains after the brief increase in temperature and humidity.

On chromosomes 2AL and 7DS, to which genes of transcription factors regulating anthocyanin biosynthesis in the pericarp of grains have been mapped, quantitative trait loci (QTLs) controlling the longevity of wheat seeds after induced senescence have been mapped too. Among such loci, for example, there are QTLs localized to regions 2AS5-0.78–1.00 and 2AL1-0.85–1.00, which contain genes affecting the production and amounts of such enzymes as NADH dehydrogenase, pyruvate decarboxylase, peroxidase, and superoxide dismutase. Genes *Per2* (peroxidase 2), *Sod* (superoxide dismutase), *Wip* (wound-induced protein), and other defense response genes of plants have been found on all three homeologous chromosomes of group 2 (Li et al., 1999). The *Cbp2* gene (chitinase-binding protein) has been mapped to the long arm of chromosome 2A (Arif et al., 2017). A QTL that controls seed longevity has also been mapped to barley chromosome 2H at a site where marker bPb6688_2H is localized, which is homologous to the gene encoding ribonuclease H (RNase H); this enzyme takes part in replication, repair, recombination, and transcription of DNA in the repair of the damage caused during seed drying in the course of ripening and subsequent storage (Nagel et al., 2015). Five DArT markers linked to QTLs controlling longevity of wheat seeds have been mapped to group 7 chromosomes in regions 7AS1-0.89–1.00, 7BS1-27-1.00, 7BL10-0.78–1.00, and 7DS4-0.61–1.00 (Arif et al., 2017). To orthologous chromosome 7H of barley, marker bPb5747_7H has been mapped, corresponding to a gene encoding a protein belonging to the ERF/APETALA2 superfamily, which is involved in plant responses to numerous stressors leading to heightened antioxidant activity (Nagel et al., 2015).

Natural seed storage and viability

Among agricultural crops, bread wheat belongs to the group of mesobiotics, the seeds of which retain germination capacity for 5–10 years under favorable storage conditions (Guryeva et al., 2021). Storage life of wheat seeds is believed to be up to 14 years under ambient conditions of 20 °C and relative humidity of up to 50 %, with a p50 value (50 % viability period) of ~7 years (Nagel and Börner, 2010).

In our work, after natural aging when seeds were stored in a dry ventilated room at 20 ± 2 °C for two, six, seven, eight, or nine years, a 50 % loss of seed viability of NILs created from the Saratovskaya 29 variety was observed after nine years of storage (Table 5), which is consistent with biological durability of grains of up to 18 years of storage.

In the present experiment, after two years of storage at 20 °C, all tested wheat samples were healthy and had 100 % seed viability and germination vigor (Tables 4 and 5). Only after six years of storage, did germination capacity of three lines – S29*Pp3Pp-D1*^{P}, S29*Pp3*^{PF}, and S29*Pp3Pp-D1*^{PF} (lines ## 4, 5, and 7) – significantly decline as compared with variety Saratovskaya 29 (line # 1, at 93 %), amounting to 83, 85, and 86 %, respectively. According to GOST R 52325-2005, germination capacity of seed material in terms of reproduction for the production of commercial products must be at least 87 % (Guryeva et al., 2021). It should be pointed out that the Saratovskaya 29 variety itself is among red-grained varieties of wheat and contains polymeric proanthocyanidins, which are synthesized in the seed coat and promote seed dormancy and longevity (Mares, Himi, 2021). It is possible that into lines carrying recombinant regions from donor varieties Purple and Purple Feed on chromosome 2AL, an allele of locus *Q.Lng.ipk.2A.1*(*SW*) has been introduced (Arif et al., 2022), which negatively affects seed lifespan.

According to the ISTA's seed testing guidelines, a reduction in germination capacity after aging, as measured using mean germination time (average latency to root emergence), is interpreted as the time required for metabolic recovery from deleterious effects of aging before germination can begin (Powell, Matthews, 2012). After seven years of storage, seeds of the S29*Pp-D1*^{P} line (# 3) – carrying a recombinant region from donor variety Purple on chromosome 7D – stood out as the most effective in terms of germination vigor and seed viability (Table 5). Significantly higher-than-normal germination capacity after nine years of storage was exhibited by seedlings from grains of isogenic lines S29*Pp-D1*^{P} and S29*Pp-D1*^{PF} (# 3 and 6), which carry recombinant regions

from variety Purple and from variety Purple Feed, respectively, on chromosome 7D. According to results of our regression analysis, the weakest slope (coefficient b_1) – and therefore the weakest influence of storage time on the germination of grains – was registered for isogenic lines S29*Pp-D1*^{P} and S29*Pp-D1*^{PF} (# 3 and 6), which carry a recombinant region from a donor variety on chromosome 7D (Table 6, Fig. 3). This result is apparently explained by genes responsible for positive regulation of seed longevity that are located in these regions of chromosome 7DS.

As reported earlier in research on traits of seed longevity in recombinant lines of wheat *Aegilops tauschii*, the chromosome 7DS region, where microsatellite marker *Xgwm1002* (linked to the *Pp-D1* gene) is located, contains loci that control the development of normal seedlings (Landjeva et al., 2010). On the other hand, the lowest germination capacity and high sensitivity to storage was observed in grains of lines with stand-alone recombinant regions on chromosome 2AL; this outcome, as we hypothesized, can be explained by negative regulation exerted by an allele of the *Q.Lng.ipk.2A.1*(*SW*) locus, which is found in this region of chromosome 2AL (Arif et al., 2022).

Germination capacity of seeds of lines S29*Pp3Pp-D1*^{P} and S29*Pp3Pp-D1*^{PF} (# 4 and 7) was also low; they have anthocyanin pigments in the pericarp and carry recombinant regions from varieties Purple Feed and Purple on chromosomes 2A and 7D. Our results revealed a neutral, and in some cases even a negative role of anthocyanins, in the caryopsis pericarp during long-term storage; this is in contrast to the findings from the testing of grains after artificial aging induced by the elevated temperature of 48 °C and 100 % humidity for 72 h. In that experiment, despite an overall decrease in germination capacity, the germination index of anthocyanin-colored grains was 20 % higher than that of lines without anthocyanin pigmentation (Table 3).

Results obtained by laboratory-based methods of artificial accelerated aging that are used to assess seed longevity under storage conditions have been questioned because these methods do not effectively simulate actual seed aging and cause considerable discrepancies in results (Schwember, Bradford, 2010; Roach et al., 2018; Gianella et al., 2022). For example, there is a report of a low correlation between grain viability after natural storage at 0 °C with 10 % relative humidity for 12–14 years and the viability of grains subjected to artificial aging (Agacka-Mołdoch et al., 2016). In this context, loci *Q.Lng.ipk-4A* and *-7B* were identified, which control the seed viability under conditions of long-term storage and artificial aging (Agacka-Mołdoch et al., 2016). In barley, QTLs responsible for grain longevity have been mapped to chromosomes 2H, 5H, and 7H (Nagel et al., 2015). It has been theorized that one of the identified loci controls the biosynthesis of glutathione, which is the most ancient redox buffer (Shvachko, Khlestkina, 2020).

It is believed that a decrease in the activity of antioxidant systems contributes to the accumulation of ROS, which is the main cause of DNA damage and deterioration of cells' condition in aged seeds, and hence their reduced germination capacity (Shvachko, Khlestkina, 2020). In ripe dry grains with a low moisture content, nucleotide mutations and degradation of macromolecules gradually accumulate as a consequence of destructive endogenous processes and metabolic by-products associated with a slowdown of repair processes during long-term storage. This notion is evidenced by the accumulation of large amounts of ROS, oxidized lipids, and aldehydes in seeds (Wiebach et al., 2020; Zhang et al., 2022). The loss of seed viability manifests itself as a decrease in the speed and uniformity of seed germination owing to a long period of pre-growth DNA repair, which begins at the earliest stages of seed impregnation with water before the start of growth and of emergence of a root through the seed coat. Cell cycle activation is regulated by checkpoint protein kinases, which slow down germination in the presence of DNA damage, and this phenomenon ultimately affects the fidelity of genetic information transfer and seed quality (Waterworth et al., 2016; Considine, Foyer, 2021). The need for prolonged repair of accumulated lesions underlies delayed germination and ultimately seed emaciation and death (Waterworth et al., 2019).

Removal of excess ROS plays a key role in the regulation of seed longevity (Zhou W. et al., 2020). Nonetheless, water-soluble anthocyanins within the grain pericarp are in a dried state and begin to perform their functions only during moistening and swelling of the seeds. Therefore, it seems that the protection of dry seeds having high cytoplasmic viscosity and low cell motility during long-term storage is carried out by other antioxidant systems, probably by glutathione (which has been detected at high concentrations in dry seeds), or by fat-soluble antioxidants. This function can be assumed for anthocyanins located in the aleuronic layer of the grain, which also contains a large amount of fatty acids. Perhaps the observed positive effect of the locus from chromosome 7DS on the viability of wheat seeds after long-term storage is explained precisely by the action of that powerful antioxidant, and not by anthocyanins, the synthesis of which is controlled by two loci, one of which (on chromosome 2A) has a negative impact on viability after long aging.

Conclusion

Thus, in this study, for the first time it was shown that anthocyanins accumulating in wheat grains have a positive effect on seed germination after artificial aging induced by elevated temperature up to 48 °C for 72 h. Under conditions of long-term natural storage, no positive effect of anthocyanins on the maintenance of seed viability was detectable. Nonetheless, the presence of a recombinant region on chromosome 7D increased the viability of seeds after long-term storage; this phenomenon may be due to the presence of loci (on this chromosome) linked with the *Pp-D1* gene, which controls wheat seeds' longevity.

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A study of the influence of the T2DL.2DS-2SS translocation and the 5S(5D) substitution from *Aegilops speltoides* on breeding-valuable traits of common wheat

R.O. Davoyan [1], I.V. Bebykina[1], E.R. Davoyan[1], A.N. Zinchenko[1], Y.S. Zubanova[1], D.M. Boldakov[1], V.I. Basov[1], E.D. Badaeva [2], I.G. Adonina [3], E.A. Salina [3]

[1] National Center of Grain named after P.P. Lukyanenko, Krasnodar, Russia
[2] N.I. Vavilov Institute of General Genetics Russian Academy of Sciences, Moscow, Russia
[3] Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
davoyanro@mail.ru

Abstract. The use of the gene pool of wild relatives for expanding the genetic diversity of common wheat is an important task of breeding programs. However, the practical application of common wheat lines with alien genetic material is constrained by the lack of information on chromosomal rearrangements and the negative impact of the transferred material on agronomically important traits. This research is aimed at studying 14 introgression lines with the T2DL.2DS-2SS translocation and the 5S(5D) substitution from *Aegilops speltoides* obtained from crossing common wheat varieties (Aurora, Krasnodarskaya 99, Nika Kubani) with the genome-substituted form Avrodes (BBAASS). Hybrid lines with different combinations of T2DL.2DS-2SS and T1BL.1RS translocations and 5S(5D) substitution were characterized by resistance to leaf and yellow rusts, productivity components and technological qualities of grain. The assessment of the varieties' resistance to rust diseases showed that Krasnodarskaya 99, Nika Kubani and the Aurora variety, which is a carrier of the T1BL.1RS translocation, are highly susceptible to diseases, while the presence of the T2DL.2DS-2SS translocation and the 5S(5D) substitution, both together and separately, provides resistance to fungal pathogens. The analysis of the lines using markers designed for known resistance genes of *Ae. speltoides* did not reveal the presence of the *Lr28*, *Lr35* and *Lr51* genes in the lines. The results suggest that the genetic material of *Ae. speltoides* transferred to chromosomes 2D and 5D contains new resistance genes. To determine the effect of the T2DL.2DS-2SS translocation and the 5S(5D) substitution on the productivity and technological qualities of grain, the lines were assessed by weight of 1000 grains, grain weight and number of ears per 1 m^2, by protein and gluten content, gluten quality and general baking evaluation. A positive effect was determined upon the weight of 1000 grains, protein and gluten content. There were no significant differences in other characteristics. The T2DL.2DS-2SS translocation and the 5S(5D) substitution did not have a negative effect on the productivity and technological quality of grain, and are of interest for breeding practice.

Key words: *Triticum aestivum*; *Aegilops speltoides*; introgressive lines; chromosomes; translocations; molecular markers; disease resistance; productivity and technological qualities of grain.

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Изучение влияния транслокации T2DL.2DS-2SS и замещения 5S(5D) от *Aegilops speltoides* на селекционно-ценные признаки мягкой пшеницы

Р.О. Давоян [1], И.В. Бебякина[1], Э.Р. Давоян[1], А.Н. Зинченко[1], Ю.С. Зубанова[1], Д.М. Болдаков[1], В.И. Басов[1], Е.Д. Бадаева [2], И.Г. Адонина [3], Е.А. Салина [3]

[1] Национальный центр зерна им. П.П. Лукьяненко, Краснодар, Россия
[2] Институт общей генетики им. Н.И. Вавилова Российской академии наук, Москва, Россия
[3] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
davoyanro@mail.ru

Аннотация. Использование генофонда диких сородичей для расширения генетического разнообразия мягкой пшеницы является актуальным направлением селекции. Однако практическое применение линий мягкой пшеницы с чужеродным генетическим материалом сдерживается ввиду отсутствия информации о хромосомных перестройках и их влиянии на важные хозяйственные признаки. Целью настоящей работы было изучение 14 интрогрессивных линий с транслокацией T2DL.2DS-2SS и замещением 5S(5D) от *Aegilops speltoides*, полученных от скрещивания сортов мягкой пшеницы Аврора, Краснодарская 99, Ника Кубани с геномно-замещенной синтетической формой Авродес (BBAASS). Гибридные линии с различным сочетанием транслокаций T2DL.2DS-2SS и T1BL.1RS и замещения 5S(5D) были охарактеризованы по устойчивости к листовой и желтой ржавчинам, компонентам продуктивности и технологическим качествам зерна. Оценка устойчивости сортов к ржавчинным болезням показала, что Краснодарская 99, Ника Кубани и сорт Аврора (носитель транслокации T1BL.1RS) высоковосприимчивы к болезням, тогда как наличие транслокации T2DL.2DS-2SS и замещения 5S(5D) как совместно, так и по отдельности обеспечивает устойчивость линий к грибным патогенам. Анализ линий с помощью маркеров, разработанных для известных генов устойчивости от *Ae. speltoides*, не выявил в линиях присутствия генов *Lr28*, *Lr35* и *Lr51*. Полученные результаты позволяют предположить, что генетический материал *Ae. speltoides* в хромосомах 2D и 5D содержит новые гены устойчивости. Для определения влияния транслокации T2DL.2DS-2SS и замещения 5S(5D) на продуктивность и технологические качества зерна проведено изучение линий по массе 1000 зерен, массе зерна и количеству колосьев с 1 м^2, содержанию белка и клейковины, качеству клейковины и общей хлебопекарной оценке. Установлен положительный эффект по массе 1000 зерен, содержанию белка и клейковины. По остальным признакам существенных различий не найдено. Транслокация T2DL.2DS-2SS и замещение 5S(5D) не оказывают негативного влияния на продуктивность и технологические качества зерна и представляют интерес для селекционной практики.
Ключевые слова: *Triticum aestivum*; *Aegilops speltoides*; интрогрессивные линии; хромосомы; транслокации; молекулярные маркеры; устойчивость к болезням; продуктивность и технологические качества зерна.

Introduction

The basis of breeding, including that of such an important agricultural crop as common wheat (*Triticum aestivum* L.), is sufficient genetic diversity. The intensification of the breeding process and the widespread distribution of varieties of the same type have led to significant genetic erosion, especially of disease resistance genes. An effective way to solve this problem is to use the gene pool of numerous species and genera related to common wheat (Knott, 1987; Friebe et al., 1996).

One of the wild relatives most widely used as a source of disease resistance is the species *Aegilops speltoides* Tausch (Manisterski et al., 1988; Kerber, Dyck, 1990). This species has given wheat genes for resistance to leaf rust – *Lr28*, *Lr35*, *Lr36*, *Lr47*, *Lr51* and *Lr66*, to stem rust – *Sr32*, *Sr39*, *Sr47*, to powdery mildew – *Pm12*, *Pm32* (McIntosh et al., 2013). *Ae. speltoides* is also characterized by its high protein content and the ability to stimulate homeologous chromosome conjugation (Dvorak, 1972). However, due to a negative impact on other economically valuable traits, introgression from this species has not been widely used in breeding practice (McIntosch et al., 1995; Helguera et al., 2005; Song et al., 2007; Brevis et al., 2008). It should be noted that the negative effect of alien introgression may depend both on the negative influence of the genetic material of the wild relative transferred along with the target gene, and on the genotypic environment of the recipient variety (Hoffann, 2008; Leonova, Budashkina, 2016).

At the "P.P. Lukyanenko National Grain Center", the genome substitution form Avrodes (BBAASS) has been developed, which is used as a "bridge" for the transfer of genetic material from *Ae. speltoides* to common wheat (Zhirov, Ternovskaya, 1984; Davoyan R.O. et al., 2012). This form exhibits high resistance to leaf rust (*Puccinia triticina* Eriks.), yellow rust (*Puccinia stiifomis* West.), powdery mildew (*Blumeria graminis* f. sp. *tritici*) and is characterized by a high protein content (Davoyan R.O. et al., 2018). This form has been involved in obtaining a large set of introgressive lines of common wheat, differing in the complex of morphological, biological and economically valuable traits, in the form of transmission of genetic material from *Ae. speltoides* (Davoyan R.O. et al., 2017).

Using the methods of differential chromosome staining (C-banding) and fluorescent *in situ* hybridization (FISH), it was found that introgressions affected mainly the chromosomes of the D genome. This is explained by the fact that in the synthetic form of Avrodes it is the D genome of common wheat that is replaced by the S genome of *Ae. speltoides*. Moreover, most of the studied lines are characterized by the T2DL.2DS-2SS translocation and the 5S(5D) substitution. To determine the breeding value of the resulting translocations and substitutions from *Ae. speltoides*, a comprehensive study of introgression lines based on economically important traits is required.

This research is aimed at the study of the impact of the T2DL.2DS-2SS translocation and the 5S(5D) substitution from *Ae. speltoides* on productivity, grain quality and resistance to fungal diseases of three varieties of common wheat of different origin.

Materials and methods

The material for the study was 14 introgressive lines of common wheat obtained from crossing the synthetic form Avrodes with the varieties susceptible to leaf and yellow rust, bred by the "P.P. Lukyanenko National Grain Center": Aurora, Krasnodarskaya 99 and Nika Kubani. Lines based on the Krasnodarskaya 99 and Aurora varieties were obtained previously (Davoyan R.O. et al., 2017) and were selected within

Table 1. Amplification conditions, names and sources of primers used to identify the *Lr28*, *Lr35*, *Lr51* genes

Genes	Primers	Annealing temperature, °C	Fragment size, bp	Reference
Lr28	CS421570-L	60	570	Cherukuri et al., 2005
	CS421570-R			
Lr35	BCD260	59	931	Seyfarth et al., 1999
	35R2			
Lr51	AGA7-759	52	819	Helguera et al., 2005
	S30-13			

the framework of this work for researching the presence of the T2DL.2DS-2SS translocations and the 5S(5D) substitution. The lines obtained on the basis of the Nika Kubani variety were characterized by cytological methods as part of this research.

Differential staining of chromosomes (C-banding) was carried out at the "N.I. Vavilov Institute of General Genetics, RAS" using a method developed in the Laboratory of Functional Chromosome Morphology of the "V.A. Engelhardt Institute of Molecular Biology, RAS" (Badaeva et al., 1994). Fluorescence *in situ* hybridization (FISH) was carried out at the "Institute of Cytology and Genetics, SB RAS" according to a previously published method (Salina et al., 2006) using probes: Spelt1 (Salina et al., 2004) to identify the genetic material of *Ae. speltoides* in the studied lines; pSc119.2 (Bedbrook et al., 1980) and pAs1 (Rayburn, Gill, 1986) to identify wheat and aegilops chromosomes (Badaeva et al., 1996; Schneider et al., 2003). The work was carried out at the Center for Microscopic Analysis of Biological Objects of the SB RAS (Novosibirsk).

Infestation of the lines was carried out under field conditions, with yellow rust in the booting phase, and with leaf rust in the boot-heading phase. In both cases, a mixture of uredospores collected from different varieties of wheat was used. The assessment was carried out when the most susceptible and late-ripening recipient variety, Aurora, reached maximum susceptibility rates (reaction type 4, degree of damage 60 %). The type of plant reaction to infection with leaf rust was determined according to the scale of E.B. Mains and H.S. Jackson (1926); to yellow rust, according to the scale of G. Gassner and U.W. Straib (1934). Plants with an intermediate type of reaction from 0 to 1 were designated as 01. The degree of plant damage was assessed using the modified Cobb scale (Peterson et al., 1948). Plants with a reaction type from 0 to 2 and a degree of damage from 0 to 20 % were classified as resistant.

DNA was isolated from 5–7-day-old etiolated wheat seedlings according to the method of J. Plaschke et al. (1995). Identification of the *Lr28*, *Lr35* and *Lr51* genes was carried out using PCR. Markers were selected according to the publication data; their names and amplification conditions are presented in Table 1.

A 25 μL reaction mixture contained 1× buffer for Taq-DNA polymerase (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 2–5 mM $MgCl_2$, 0.01 % Tween-20), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 12.5 mM of each primer, 50 ng DNA and 1 unit of Taq polymerase. Amplification was carried out according to the conditions given in Table 1. PCR products for the *Lr28* and *Lr35* genes were separated using electrophoresis in a 1.8 % agarose gel with 0.5× TBE buffer; in the case of the *Lr51* gene, a 3 % agarose gel was used with MS-12 agarose, Molecular Screening "diaGene" with increased clarity of fragment separation. DNA marker M24 100 bp "SibEnzyme" was used as a molecular weight marker. Gels were stained with ethidium bromide and photographed under ultraviolet light using an Infiniti 1000 photobox.

To characterize the lines by productivity, the weight of 1000 grains, grain weight and the number of ears per plot were determined. The plot area was 1 m^2, there were four replications. The technological qualities of grain and flour were studied in the Department of Technology and Biochemistry of Grain of the "P.P. Lukyanenko National Grain Center" according to the methods of the State Variety Testing of Agricultural Crops (1988). Statistical processing of the obtained results was carried out using the AGROS-2.10 program.

Results

To determine the breeding value of the T2DL.2DS-2SS translocation and the 5S(5D) substitution from *Ae. speltoides,* a study was carried out on 14 introgressive lines obtained with the participation of three varieties susceptible to leaf and yellow rust: Aurora, Krasnodarskaya 99 and Nika Kubani. The characteristics of the lines concerning introgressions and resistance to leaf and yellow rusts are given in Table 2.

The majority of the presented lines are characterized by a combination of the T2DL.2DS-2SS translocation and the 5S(5D) chromosomal substitution (Table 2, Fig. 1). Also, a significant number of the lines have the T1BL.1RS translocation (Table 2, Fig. 1), obtained from the synthetic form of Avrodes. In line 1889n17, a single T2DL.2DS-2SS translocation was detected (Fig. 2*a*). Only a 5S(5D) chromosomal substitution was detected in lines 1009n19 and 493n20 (Fig. 2*b*).

Recipient varieties Aurora, Nika Kubani and Krasnodarskaya 99 are susceptible to leaf and yellow rust. The T2DL.2DS-2SS translocation and the 5S(5D) substitution, both together and separately, provide line resistance to these pathogens (Table 2). Line 1889n17 with the T2DL.2DS-2SS

Table 2. The characteristics of the *T. aestivum*/Avrodes lines concerning introgressions and resistance to leaf and yellow rusts

Line	*T. aestivum* (recipient variety)	Translocation, substitution	Type of reaction and degree of damage, score/%	
			leaf rust	yellow rust
D37n10	Aurora	T1BL.1RS, T2DL.2DS-2SS, 5S(5D)	01/10	2/10
AA60n9	Aurora	T1BL.1RS, T2DL.2DS-2SS, 5S(5D)	01/10	2/20
1575n17	Aurora	T1BL.1RS, T2DL.2DS-2SS, 5S(5D)	1/10	1/5
3210n15	Krasnodarskaya 99	T2DL.2DS-2SS, 5S(5D)	01/5	1/10
3198n15	Krasnodarskaya 99	T1BL.1RS, T2DL.2DS-2SS, 5S(5D)	01/5	1/10
3193n15	Krasnodarskaya 99	T2A, T1D, T2DL.2DS-2SS, 5S(5D)	01/5	1/5
2900n17	Krasnodarskaya 99	T1BL.1RS, T2DL.2DS-2SS, 5S(5D)	1/10	1/10
2955n17	Krasnodarskaya 99	T1BL.1RS, T2DL.2DS-2SS, 5S(5D)	01/5	1/10
2636n18	Krasnodarskaya 99	T2DL.2DS-2SS, 5S(5D)	1/5	1/5
1009n19	Krasnodarskaya 99	5S(5D)	2/20	2/10
95n20	Krasnodarskaya 99	T1BL.1RS, 5S(5D)	2/20	1/10
1889n17	Nika Kubani	T2DL.2DS-2SS	1/10	1/5
1249n19	Nika Kubani	T1BL.1RS, T2DL.2DS-2SS, 5S(5D)	01/5	1/5
493n20	Nika Kubani	5S(5D)	2/10	1/10
Aurora		T1BL.1RS	4/60	4/60
Krasnodarskaya 99			4/80	3/40
Nika Kubani			3/60	4/60

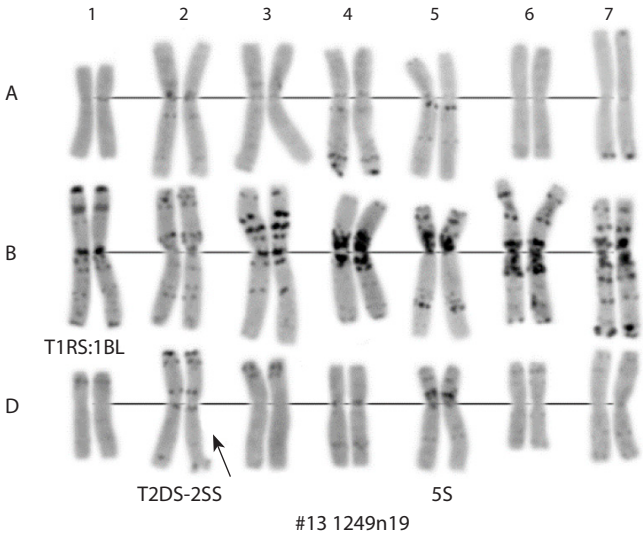

Fig. 1. Differentially stained karyotype of line 1249n19.

translocation exhibits higher resistance to leaf rust (reaction type 1, severity of damage 10 %) compared to lines 1009n19, 95n20 and 493n20 with the 5S(5D) substitution.

Since one of the main objectives was the transfer of resistance to leaf rust from the synthetic form Avrodes, genes for resistance to this disease were identified using DNA markers. Among the known, identified leaf rust resistance genes derived from *Ae. speltoides*, the effective gene *Lr35* was found in Avrodes (Davoyan E.R et al., 2012) (Fig. 3*a*), as well as the genes *Lr28* and *Lr51* (Fig. 3*b* and 3*c*, respectively). Since the absence of the *Lr28* and *Lr35* genes in the AA60n9 line was previously determined (Davoyan R.O. et al., 2017), in this research this line was studied for the presence of only the *Lr51* gene. There were no *Lr28*, *Lr35* and *Lr51* genes found in the studied lines (Fig. 3*a*, *2–4*, *6*, *7*, *9–17*; Fig. 3*b*, *4–8*, *10–15*, *17*; 3*c*, *4–17*).

To determine the breeding value of the T2DL.2DS-2SS translocation and the 5S(5D) substitution, the lines were assessed for productivity components and technological qualities of grain and flour.

Productivity was determined by the weight of 1000 grains, the weight of grains and the number of ears per 1 m^2 (Table 3). In the lines obtained with the participation of the Aurora variety as a recipient, a significant excess in the weight of 1000 grains was revealed. The highest value for this indicator had line 1575n17 (41.7 g). There were no significant differences in the number of formed ears per 1 m^2. In terms of grain weight per 1 m^2, lines D37n10 and AA60n9 were at the same level, and line 1575n17 was significantly higher than the Aurora variety.

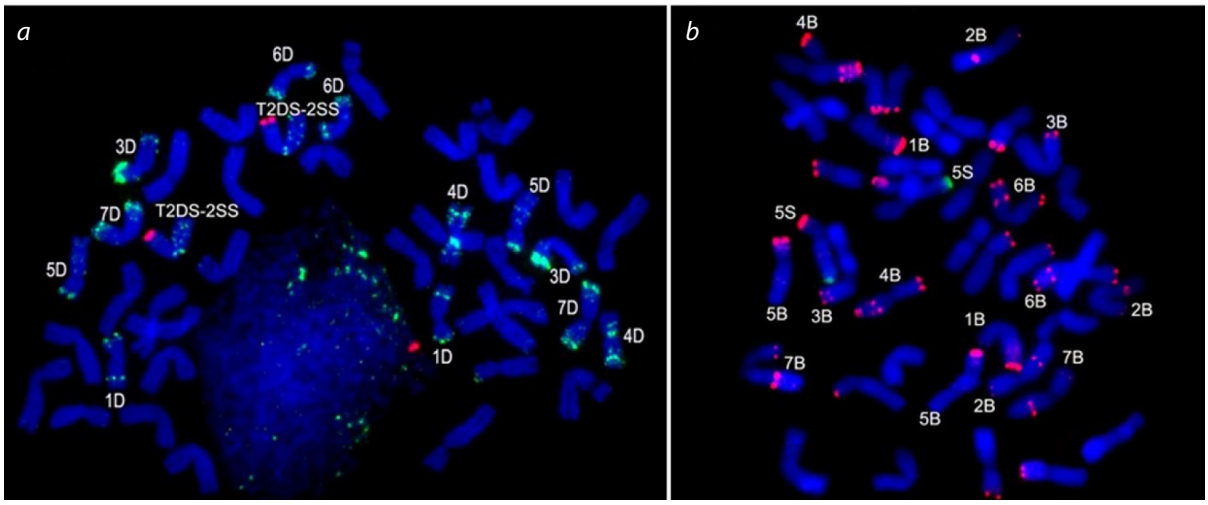

Fig. 2. FISH results on metaphase chromosomes of lines: (*a*) 1889n17 with probes pAs1 (green) and Spelt1 (red); (*b*) 493n20 with probes pSc119.2 (red) and Spelt1 (green).

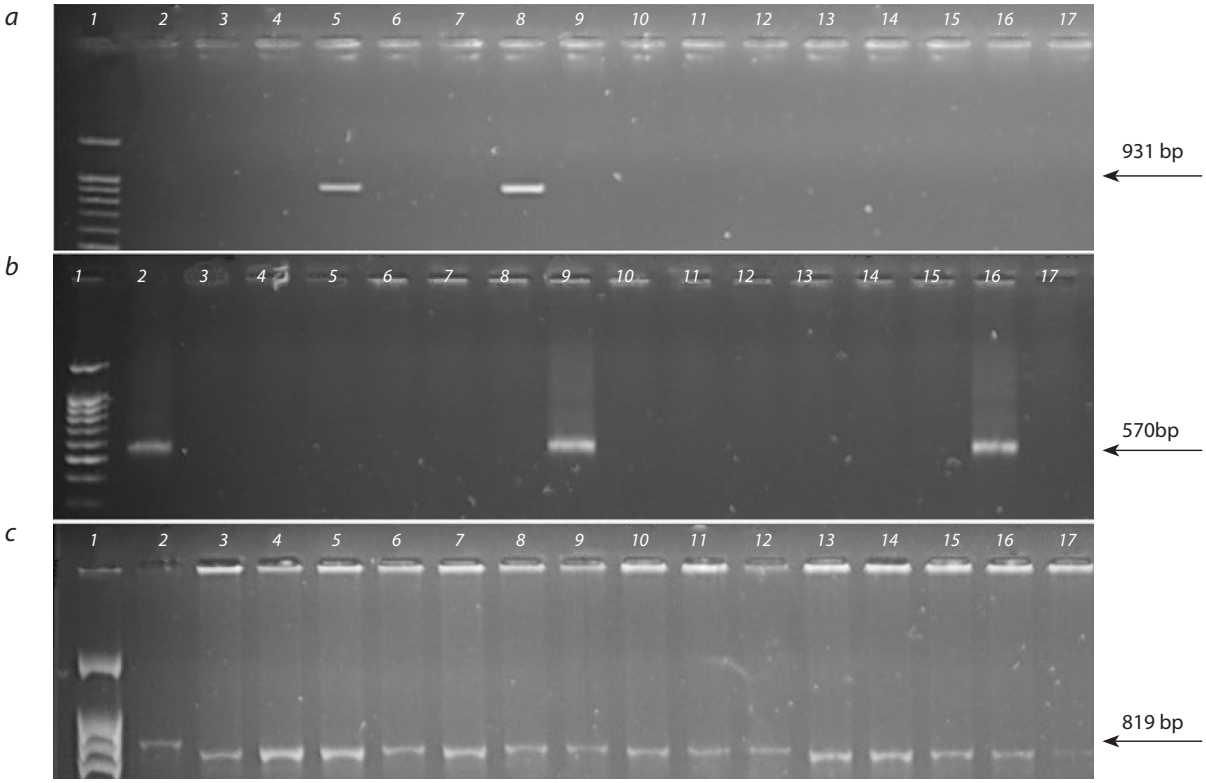

Fig. 3. Electropherograms of amplification products using primers to diagnostic markers linked to genes: *a*) *Lr35* (*1* – length marker, *2* – Aurora, *5* – TcLr35, *8* – Avrodes; *2–4*, *6*, *7*, *9–17* – introgression lines); *b*) *Lr28* (*1* – length marker, *2* – TcLr28, *9*, *16* – Avrodes, *3* – Aurora, *4–8*, *10–15*, *17* – introgression lines); *c*) *Lr51* (*1* – length marker, *2* – Avrodes, *3* – Aurora, *4–17* – introgression lines).

All lines obtained with the participation of the Krasnodarskaya 99 variety reliably exceeded it in the weight of 1000 grains. There were no significant differences in the number of ears per 1 m^2 and grain weight.

Line 1249n19 significantly exceeded the recipient variety Nika Kubani in the weight of 1000 grains (40.2 g) and the weight of grain per 1 m^2 (570.5 g). The weight of 1000 grains for lines 1889n17 and 493n20 was also higher than that of Nika Kubani. The differences in the number of ears and grain weight per 1 m^2 were insignificant.

Important features that limit the use of lines carrying alien genetic material in breeding practice are the technological cha-

Table 3. Productivity components of introgressive lines of *T. aestivum*/Avrodes

Line	*T. aestivum* (recipient variety)	Weight of 1000 grains, g	Number of ears per 1 m^2, pcs.	Grain weight per 1 m^2, g
D37n10	Aurora	40.3	345.2	435.4
AA60n9	Aurora	40.9	331.7	448.2
1575n17	Aurora	41.7	352.3	528.5
3210n15	Krasnodarskaya 99	39.3	437.2	558.3
3198n15	Krasnodarskaya 99	40.2	474.2	570.2
3193n15	Krasnodarskaya 99	41.1	452.9	543.4
2900n17	Krasnodarskaya 99	40.5	450.7	537.9
2955n17	Krasnodarskaya 99	39.7	461.5	542.8
2636n18	Krasnodarskaya 99	40.3	452.7	527.5
1009n19	Krasnodarskaya 99	39.8	462.7	556.2
95n20	Krasnodarskaya 99	41.1	447.4	550.3
1889n17	Nika Kubani	39.4	443.6	540.8
1249n19	Nika Kubani	40.2	410.2	570.5
493n20	Nika Kubani	40.9	425.8	528.4
Aurora		39.4	357.0	410.7
Krasnodarskaya 99		38.1	456.2	531.3
Nika Kubani		38.7	430.4	510.8
$LSD_{0.5}$		0.6	28.6	40.7

Note. LSD – least significant difference.

racteristics of grain. The lines obtained with the participation of the Aurora variety as a recipient have similar characteristics of protein and gluten content, gluten quality and general baking assessment (Table 4).

The transfer of the T2DL.2DS-2SS translocation and the chromosomal 5S(5D) substitution to the Krasnodarskaya 99 variety contributed to an increase in protein and gluten content in the lines (Table 4). The excess protein content in lines 3210n15, 2955n17, 2636n18 and 1009n19 ranged from 2 to 3 %. At the same time, it should be noted that all lines have higher GDI values and correspond to the second group of GOST in terms of gluten quality. The lines are also inferior to the Krasnodarskaya 99 variety in terms of volumetric bread yield and overall baking rating. Lines 1249n19 and 493n20 have approximately the same performance as the recipient variety Nika Kubani. Line 1889n17 exceeds the recipient variety in protein and gluten content, but is inferior to it in gluten quality (Table 4).

Discussion

The transfer of economically valuable genes from the gene pool of numerous related species and genera to common wheat remains an effective way of solving current breeding problems.

The purpose of using the synthetic form Avrodes, first of all, was the transfer of new genes for resistance to diseases, in particular to leaf rust, from *Ae. speltoides* to common wheat. Currently, the catalog of wheat gene symbols includes six resistance genes transmitted from this species: *Lr28*, *Lr35*, *Lr36*, *Lr47*, *Lr51* and *Lr66* (McIntosh et al., 2013), respectively localized in common wheat chromosomes 4A, 2B, 6B, 7A, 1B and 3A (Friebe et al., 1996). Additionally, I.G. Adonina et al. (2012) characterized the T5BS.5BL-5SL translocation from *Ae. speltoides* with an effective gene designated *LrASP5*.

Our molecular genetic analysis did not reveal in the studied wheat lines the presence of effective resistance genes *Lr28*, *Lr35* and *Lr51* present in the synthetic Avrodes. We found that the T2DL.2DS-2SS translocation and the 5S(5D) substitution from *Ae. speltoides,* both together and separately, provide wheat lines with resistance to leaf rust. At the same time, line 1889n17 with the T2DL.2DS-2SS translocation is characterized by higher resistance to leaf rust (reaction type 1) than lines with only the 5S(5D) substitution (reaction type 2) (Table 2). None of the previously transferred known leaf rust resistance genes are located on chromosomes 2D and 5D. According to S.N. Sibikeev et al. (2015), the 2D/2S translocation is carried by lines L195 and L200, which are resistant to leaf and stem rust. Due to the lack of these lines at our disposal, we were unable to clarify the identity of these leaf rust resistance genes with the genes present in the lines we obtained.

Table 4. Technological characteristics of introgressive lines of *T. aestivum*/Avrodes

Line	Recipient variety	Protein content, %	Gluten content, %	GDI (units)	Volume yield of bread, ml	General score, point
D37n10	Aurora	15.9	24.0	86	680	4.2
AA60n9	Aurora	16.1	29.1	85	700	4.3
1575n17	Aurora	15.0	27.8	90	720	4.1
3210n15	Krasnodarskaya 99	16.4	32.2	80	770	4.2
3198n15	Krasnodarskaya 99	15.2	28.7	86	700	4.3
3193n15	Krasnodarskaya 99	15.6	29.6	85	760	4.5
2900n17	Krasnodarskaya 99	16.3	28.5	85	720	4.3
2955n17	Krasnodarskaya 99	15.9	31.3	93	670	4.3
2636n18	Krasnodarskaya 99	15.8	29.3	82	750	4.5
1009n19	Krasnodarskaya 99	16.1	30.8	91	690	4.1
95n20	Krasnodarskaya 99	15.2	29.1	88	740	4.0
1889n17	Nika Kubani	15.4	31.1	87	780	4.3
1249n19	Nika Kubani	14.0	26.1	72	650	4.2
493n20	Nika Kubani	14.5	27.3	83	765	4.1
Aurora		15.7	29.8	84	700	4.3
Krasnodarskaya 99		13.8	26.0	65	800	4.6
Nika Kubani		14.5	28.7	74	770	4.3
$LSD_{0.5}$		0.3	0.8	1.4	10.2	–

Note. GDI – gluten deformation index.

It should also be noted that our lines with the T2DL.2DS-2SS translocation and the 5S(5D) substitution are resistant to yellow rust, which is one of the most common wheat diseases. Although until the end of the 1960s it had no economic significance on the territory of Russia, since 1990, in the south, primarily in the Krasnodar region, the yellow rust pathogen has had a tendency of expanding its range, and the damage to some varieties of winter wheat against a natural infectious background has reached 90 % (Volkova et al., 2020). At the same time, not a single yellow rust resistance gene transferred to the wheat genome from *Ae. speltoides* (McIntosh et al., 2013) is registered in the catalog of gene symbols. Thus, our results indicate the possible transfer of new resistance genes to common wheat from this species. Additional research is necessary to test this assumption.

When transferring alien genetic material, along with useful traits (disease resistance, high protein content, etc.), introgressions often have a negative impact on the productivity and technological characteristics of grain. For this reason, a number of alien translocations have not found wide application in breeding practice. Thus, of the abovementioned six resistance genes to leaf rust, only the *Lr28* and *Lr47* genes are used in practical breeding (Leonova, 2018). At the same time, introgression lines with genetic material of *Ae. speltoides* can combine disease resistance with productivity and good technological qualities of grain and flour (Lapochkina et al., 1996; Sibikeev et al., 2015; Davoyan R.O. et al., 2017).

Based on our results (Table 3), we can conclude that the presence of the T2DL.2DS-2SS translocation and even the 5S(5D) chromosomal substitution in the lines does not have a negative effect on the elements of productivity. Two lines, 1575n17 and 1249n19, significantly exceed the recipient varieties Aurora and Nika Kubani, respectively, in terms of grain weight per 1 m^2. In the remaining lines, no significant differences were found either in the number of ears per 1 m^2 or in grain weight. A positive effect of the T2DL.2DS-2SS translocation and the 5S(5D) substitution on the weight of 1000 grains was determined. Almost all the lines we studied exceeded the recipient varieties for this trait, while, for example, in the work of N.V. Petrash et al. (2016), a decrease in the weight of 1000 grains was noted in all introgressive lines, regardless of chromosomal localization (chromosomes 5BL, 6BL and 7D) of alien chromatin.

The study of the technological characteristics of grain and flour also did not reveal a negative effect of the T2DL.2DS-2SS translocation and the 5S(5D) substitution (Table 4). There were no significant differences in protein and gluten content, gluten quality and overall baking rating between the Aurora

variety and the lines obtained on its basis. The lines obtained with the Krasnodarskaya 99 variety, in comparison with it, have higher levels of protein and gluten content and, despite a slight deterioration in the quality of gluten (second group of GOST), in general, they received a fairly high baking rating. The technological characteristics of the Nika Kubani/Avrodes lines are similar to those for the recipient variety Nika Kubani.

The manifestation of traits in introgressive lines depends not only on the alien genetic material present in them, but also on the genotypic environment of the recipient variety. In our studies, the nature of the manifestation of the T2DL.2DS-2SS translocation and the 5S(5D) substitution was studied on the genetic background of three common wheat varieties of different origins. The lines combine disease resistance with good indicators of productivity, grain and flour quality, regardless of the recipient variety.

Conclusion

Thus, we can conclude that the resulting new translocation T2DL.2DS-2SS and the substitution 5S(5D) from *Ae. speltoides* can contribute to the improvement of common wheat, in particular in terms of disease resistance, protein and gluten content, as well as weight of 1 000 grains, and are of interest for breeding practice.

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Identification of quantitative trait loci of pod dehiscence in a collection of soybean grown in the southeast of Kazakhstan

B.N. Doszhanova [1, 2], A.K. Zatybekov [1], S.V. Didorenko [3], T. Suzuki [4], Y. Yamashita [4], Y. Turuspekov [1, 2]

[1] Institute of Plant Biology and Biotechnology, Almaty, Kazakhstan
[2] Al-Farabi Kazakh National University, Almaty, Kazakhstan
[3] Kazakh Research Institute of Agriculture and Plant Growing, Almalybak, Almaty region, Kazakhstan
[4] Hokkaido Research Organization, Sapporo, Japan

yerlant@yahoo.com

Abstract. Soybean [*Glycine max* (L.) Merr.] is one of the important crops that are constantly increasing their cultivation area in Kazakhstan. It is particularly significant in the southeastern regions of the country, which are currently predominant areas for cultivating this crop. One negative trait reducing yield in these dry areas is pod dehiscence (PD). Therefore, it is essential to understand the genetic control of PD to breed new cultivars with high yield potential. In this study, we evaluated 273 soybean accessions from different regions of the world for PD resistance in the conditions of southeastern regions of Kazakhstan in 2019 and 2021. The field data for PD suggested that 12 accessions were susceptible to PD in both studied years, and 32 accessions, in one of the two studied years. The genotyping of the collection using a DNA marker for the *Pdh1* gene, a major gene for PD, revealed that 244 accessions had the homozygous *R* (resistant) allele, 14 had the homozygous *S* (susceptible) allele, and 15 accessions showed heterozygosity. To identify additional quantitative trait loci (QTLs), we applied an association mapping study using a 6K SNP Illumina iSelect array. The results suggested that in addition to major QTL on chromosome 16, linked to the physical location of *Pdh1*, two minor QTLs were identified on chromosomes 10 and 13. Both minor QTLs for PD were associated with calmodulin-binding protein, which presumably plays an important role in regulating PD in dry areas. Thus, the current study provided additional insight into PD regulation in soybean. The identified QTLs for PD can be efficiently employed in breeding for high-yield soybean cultivars.

Key words: soybean; pod dehiscence; seed yield; genome-wide association study; quantitative trait locus.

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Идентификация количественных локусов признака растрескивания бобов в коллекции сои, выращенной на юго-востоке Казахстана

Б.Н. Досжанова [1, 2], А.К. Затыбеков [1], С.В. Дидоренко [3], Т. Сузуки [4], Й. Ямашита [4], Е.К. Туруспеков [1, 2]

[1] Институт биологии и биотехнологии растений, Алматы, Казахстан
[2] Казахский национальный университет им. аль-Фараби, Алматы, Казахстан
[3] Казахский научно-исследовательский институт земледелия и растениеводства, пос. Алмалыбак, Алматинская область, Казахстан
[4] Научно-исследовательская организация Хоккайдо, Саппоро, Япония

yerlant@yahoo.com

Аннотация. Соя [*Glycine max* (L.) Merr.] – одна из важнейших сельскохозяйственных культур, площади которой в Казахстане постоянно увеличиваются. Особенно эта культура значима в южных и юго-восточных регионах страны, которые являются основными регионами выращивания сои. К негативным факторам, влияющим на урожайность сои в засушливых районах, относится растрескивание стручков. Поэтому понимание генетического механизма растрескиваемости стручков сои важно для выведения новых высокоурожайных сортов. В настоящем исследовании мы изучили 273 сорта и линии сои из разных регионов мира на устойчивость к растрескиваемости в условиях Южного Казахстана в 2019 и 2021 гг. Наблюдения за признаком «растрескиваемость стручков сои» в полевых условиях Алматинской области выявили, что в 2019 г. растрескиванию были подвержены 23 сорта, в 2021 г. – 21 сорт. Двенадцать сортов сои повторно подвергались растрескиванию в оба года эксперимента. Согласно средним данным испытаний, всего подвержены растрескиванию 32 сорта сои. При генотипировании коллекции с использованием ДНК-маркера гена *Pdh1*, основного гена растрескиваемости стручков сои, у 244 образцов был выявлен устойчивый аллель, у 14 образцов – восприимчивый, а 15 образцов обладали гетерозиготностью. Для идентифи-

кации дополнительных локусов количественных признаков (quantitative trait locus, QTL) мы применили полногеномный анализ с использованием 6 тысяч SNP-маркеров на основе чипа 6K SNP Illumina iSelect. В дополнение к основному QTL на хромосоме 16, связанному с физическим расположением гена *Pdh1*, были идентифицированы два минорных QTL на хромосомах 10 и 13. Оба минорных локуса ассоциированы с растрескиванием стручков сои и связаны с кальмодулин-связывающим белком, который, вероятно, играет важную роль в регулировании растрескиваемости стручков сои в засушливых регионах. Таким образом, нами получена дополнительная информация о регуляции растрескиваемости в сое. Идентифицированные QTL для признака «растрескиваемость стручков сои» могут быть эффективно использованы при селекции высокоурожайных сортов сои.

Ключевые слова: соя; растрескивание бобов; урожай зерна; полногеномный анализ; локусы количественных признаков; QTL.

Introduction

Soybean [*Glycine max* (L.) Merr.] is a major crop among oilseeds worldwide and a global source of edible protein and oil, providing approximately 60 and 28 % of the world supply, respectively (Vollmann et al., 2000; Zhou et al., 2020). According to the USDA, Brazil, the United States of America, and Argentina are the largest soybean production countries, while Kazakhstan is on the list of the top forty producers (https://ipad.fas.usda.gov). Kazakhstan is one of the largest agro-industrial countries in Central Asia and is interested in increasing soybean production areas (Abugalieva et al., 2016; Didorenko et al., 2016; Zatybekov et al., 2017). Therefore, developing new competitive cultivars for new cultivation areas is a priority for the local breeding community.

One of the limiting factors for the increase in soybean productivity, particularly in southern regions, is pod dehiscence (PD), which leads to a substantial yield loss (Zhang Q. et al., 2018). For wild plants, PD is an important mechanism for spreading progenies (Benvenuti, 2007; Fuller, 2007), but for cultivated plants, it is an unfavorable agronomic trait because mature pods open to release seeds before harvesting (Kang et al., 2009; Zhang L., Boahen, 2010). PD was nearly eliminated during soybean domestication and breeding (Liu et al., 2007; Krisnawati, Adie, 2017). Nevertheless, the yield losses due to PD today may range from 34 to 99 % depending on genetic background, environmental factors, pod morphology and anatomy, and management (Romkaew, Umezaki, 2006; Bhor et al., 2014; Parker et al., 2021).

Pod dehiscence is a highly heritable and complex trait; it was shown that its broad sense heritability may range from 90 to 98 % in different populations (Tsuchiya, 1987; Bailey et al., 1997; Kang et al., 2009). Previously, two genes, *Pdh1* and *SHAT1-5*, were identified and mapped on chromosome 16 (Funatsuki et al., 2008, 2014; Dong et al., 2014). The gene *pdh1* was identified in cultivated soybeans by Funatsuki and co-authors in 2014 (Funatsuki et al., 2014). The dominant *Pdh1* encodes a dirigent family protein in soybean and is highly expressed in the pod endocarp layer, increasing dehiscing forces. The recessive *pdh1* in dehiscence-resistant types includes a premature stop codon, which blocks proper protein synthesis (Funatsuki et al., 2014). The effect of *pdh1* on pod dehiscence is generally larger among the other genes that had important value in worldwide soybean cultivation (Funatsuki et al., 2014; Hu et al., 2019; Zhang J., Singh, 2020). *SHAT1-5* gene activates secondary wall synthesis and stimulates the dehiscence site's thickening in pods. The domestication process resulted in extra *SHAT1-5* expression compared to the wild soybean allele (Dong et al., 2014). Previous research suggested that all domesticated soybeans carry *SHAT1-5* haplotypes derived from a haplotype that differs from wild soybeans (Funatsuki et al., 2014; Sedivy et al., 2017).

Recently, a genome-wide association study (GWAS) described another dehiscence-associated candidate gene, Glyma09g06290 (Hu et al., 2019). This gene is highly expressed in developing pods; however, the biological functions of this gene should be further investigated (Hu et al., 2019). Later, another GWAS showed that the *NST1A* gene (Glyma.07G050600) has a potential role in soybean pod dehiscence (Zhang J., Singh, 2020). *NST1A* codes a NAC family transcription factor and a paralog of *SHAT1-5* (NAC are NAM, ATAF1/2, and CUC2 proteins, the largest families of transcription factors in plants: NAM – no apical meristem proteins, ATAF1/2 – Arabidopsis transcription activation factor, CUC2 – cup-shaped cotyledon; NST1-NAC secondary thickening1) (Zhang J., Singh, 2020). The authors identified an indel in its coding sequence, leading to a premature stop codon. Epistatic analyses showed that *NST1A* works with *Pdh1* to provide durable resistance to pod dehiscence (Zhang J., Singh, 2020; Parker et al., 2021).

Apart from genes, several QTLs were repeatedly identified throughout the soybean genome on different chromosomes. To date, several QTLs for PD have been identified on almost all chromosomes in different soybean populations (Bailey et al., 1997; Liu et al., 2007; Kang et al., 2009; Yamada et al., 2009; Han et al., 2019; Hu et al., 2019). The identified QTL on chromosome 16 was located near the major gene *pdh1* and had a high value of the coefficient of determination (Seo et al., 2020; Jia et al., 2022).

Most new QTLs were identified using GWAS, a powerful tool for detecting natural variation involving the regulation of complex traits based on genotype-phenotype association (Rafalski, 2010; Huang, Han, 2014). Although many QTLs for PD in soybeans were discovered, some can be unstable in different environments and may vary in diverse genetic backgrounds (Hu et al., 2019; Seo et al., 2020; Jia et al., 2022). Hence, additional studies for searching QTLs for PD are important for breeding practices in new soybean environments. Therefore, this study aimed to identify QTLs for PD in the southeast region of Kazakhstan using a diverse world soybean collection.

Materials and methods

Field evaluation of the collection. The soybean collection consisted of 273 cultivars and lines from Eastern and Western European countries, North America, and East and Central Asia (Supplementary Material 1)[1] (Zatybekov et al., 2017, 2018).

[1] Supplementary Materials 1–5 are available at:
https://vavilovj-icg.ru/download/pict-2024-28/appx19.pdf

The collection was grown in 2019 and 2021 at the experimental stations of Kazakh Research Institute of Agriculture and Plant Growing (KRIAPG, Almaty region, Kazakhstan) located at an altitude of 740 m above sea level, 43°15′ N, 76°54′ W (Doszhanova et al., 2019). This site is characterized by continental climatic conditions: mild and cool winters, cool spring, hot and dry summers, and warm and dry fall. The meteorological data registered for the experiments are provided in Supplementary Material 2. The collection was planted in four rows per plot, 25 cm plant spacing, 50 cm row spacing, and 1 m row length without soil fertilizers.

The yield component traits screened in soybean accessions are the number of fruiting nodes (NFN, pcs), the number of seeds per plant (NSP, pcs), yield per plant (YP, g), thousand seed weight (TSW, g). The PD data was collected by visually estimating the percentage of pods at the R8 stage in a plot that had dehisced at the full maturity stage on a scale of 1–5, where 1 ≤ 1–20 %, 2 ≤ 21–40 %, 3 ≤ 41–60 %, 4 ≤ 61–80 % and 5 ≤ 81–100 % (Supplementary Material 1). Correlation analysis was conducted using RStudio software (Allaire, 2011).

DNA extraction and PCR procedure. DNA was extracted from young leaves by a modified CTAB method (Suzuki et al., 2012). Amplification of DNA was performed using an allele-specific PCR method with four primers for the SNP marker of the *Pdh1* gene associated with pod dehiscence in soybean (Funatsuki et al., 2014). PCR reaction of 10 µl of the solution containing the DNA template (50 ng/µl), AmpliTaqGold MasterMix (Applied Biosystems by Thermo Fisher Scientific), two pairs of primers (forward and reverse outer primers, forward and reverse inner primers), and M13 primer, labeled with fluorescent (FAM, NED, VIC and PET, Applied Biosystems). PCR amplification used an initial 95 °C for 7 min; 35 cycles of 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 1 min, and a final 72 °C extension for 7 min. PCR products were analyzed on an ABI Prism 3500 Genetic Analyzer (Applied Biosystems) with GeneMapper software as described previously (Suzuki et al., 2012).

Linkage disequilibrium, population structure, and genome-wide association study. For GWAS, the genomic DNA of all samples in the collection was genotyped using the 6K SNP Illumina iSelect array (Song et al., 2013) at the Trait Genetics Company (TraitGenetics GmbH Gatersleben, Germany). SNP genotype analysis was carried out using Illumina Genome Studio software (GS V2011.1). The quality control of genotyped data was performed by filtering SNPs with call rate ≥90 % and minor allele frequency (MAF) ≥5 %. Accessions with missing data being greater than 10 % were removed. SNP loci with more than 10 % heterozygous calls were also removed (Bradbury et al., 2007). Pairwise linkage disequilibrium (LD) between the markers based on their correlations (R2) was calculated using TASSEL. R statistical software was used to plot the correlation between pairwise R2 and the genetic distance, LD decay plot (www.R-project.org).

The population structure (Q) analysis was performed using STRUCTURE software version 2.3.4 (Pritchard et al., 2000). The optimal number of clusters (K) was chosen based on the ΔK as described by (Evanno et al., 2005). The obtained values were then transformed into a population structure (Q) matrix. The kinship matrix (K) was generated by TASSEL software V5.0 (Bradbury et al., 2007).

GWAS was conducted based on the Mixed Linear Model (Q + K) using TASSEL software V5.0 (Bradbury et al., 2007). The statistical significance thresholds, Bonferroni correction, and alternative method False Discovery Rate (FDR) were used to distinguish true positives from false positives and false negatives. The significance level of 5 % after Bonferroni multiple test correction was used to identify significant associations (Buckler et al., 2011). The Benjamini–Hochberg procedure was calculated to control the FDR threshold at 5 % (Benjamini, Hochberg, 1995). The SoyBase database (www.soybase.org) was used to search genes for identified marker-trait associations.

Results

Field experiments and traits evaluation

Observing PD in the field conditions of the Almaty region showed that 23 accessions in 2019 and 21 accessions in 2021 dehisced their pods in the field conditions (Fig. 1), and 12 accessions repeatedly fully or almost fully dehisced their pods with grade 4 or 5 in two years of experiments in the Almaty region (Supplementary Material 1).

The results of two years of experiments showed that the vast majority of the soybean collection was resistant to PD in the Almaty region conditions, but 32 accessions were found to be susceptible to PD in one of the two years of study. After harvesting, the soybean collection was analyzed by yield components, such as NSP, NFN, YP, and TSW. The soybean collection studied in the Almaty region was more productive in 2021 than in 2019. The average values of two years for NFN, NSP, YP, and TSW were 15.01 nodes, 37.88 seeds, 9.62 g, and 149.12 g, respectively. The ranges of soybean yield components in the Almaty region in two experimental years and average data are shown in Table 1.

Pearson correlation analysis suggested that the average data of the PD trait in the field conditions of the Almaty region were negatively and significantly associated with all yield com-

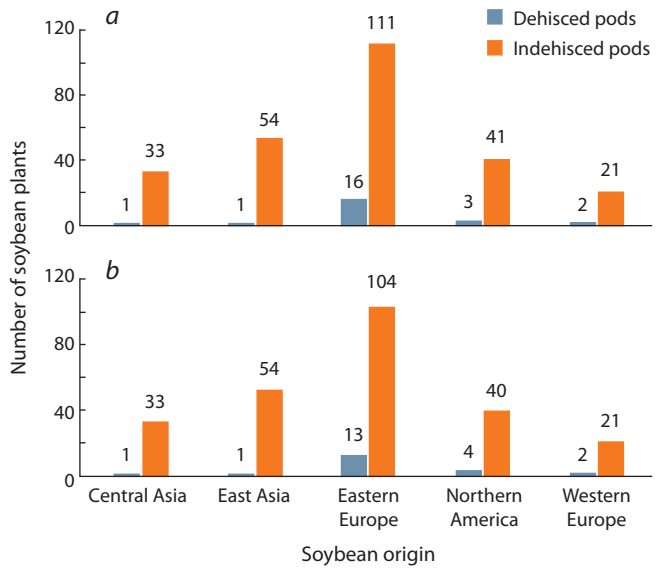

Fig. 1. The field screening of the world soybean collection by the pod dehiscence trait in 2019 (*a*) and 2021 (*b*) years of experiments.

Table 1. The variability ranges of yield component traits in 2019, 2021, and the average of two years

Year	Ranges	NFN	NSP	YP	TSW
2019	Max	31.3	87.7	39.3	310.9
	Min	4.9	8.4	1.5	18.9
	Mean ± SE	14.38 ± 0.33	36.28 ± 0.95	10.38 ± 0.34	124.28 ± 3.38
2021	Max	52	126	55.6	276
	Min	3.7	6.3	0.3	118
	Mean ± SE	15.63 ± 0.44	39.43 ± 1.25	8.85 ± 0.41	173.18 ± 1.51
Average	Max	34.05	81.85	31.5	228.4
	Min	5.2	10.5	1.95	97.05
	Mean ± SE	15.01 ± 0.35	37.88 ± 1.01	9.62 ± 0.32	149.12 ± 1.63

Note. NFN – number of fruiting nodes (pcs), NSP – number of seeds per plant (pcs), YP – yield per plant (g), TSW – thousand seed weight (g), SE – standard error.

Fig. 2. Correlation analysis of the pod dehiscence trait in the field conditions and yield components.

Field PD – pod dehiscence in the field conditions.

ponents, NFN, NSP, YP, and TSW, with coefficients of correlation –0.27, –0.29, –0.2, and –0.27 respectively ($p < 0.01$, RStudio). In their turn, NSP, YP, and TSW had a significant and positive correlation with each other ($p < 0.01$) (Fig. 2).

Genotyping of soybean collection

The soybean collection consisted of 273 samples and was genotyped using four primers for the SNP marker of the *Pdh1* gene, which is associated with PD. The SNP analysis of soybean accessions identified three alleles: *S* – pod dehiscence susceptible, *R* – pod dehiscence resistant, and *H* – heterozygous (Fig. 3). A *t*-test with significance confirmed the difference among groups of three alleles at $p < 0.001$.

The results of *Pdh1* genotyping using an allele-specific SNP marker showed that 244 out of 273 accessions were with the homozygous *R* (resistant) allele, 14 had the homozygous *S* (susceptible) allele, and 15 samples were heterozygotes (Supplementary Material 1). Figure 4 illustrates the distribution of alleles of different origins in the soybean collection.

Most of the accessions carrying the susceptible *S* alleles in homozygous or heterozygous genotypes were from Eastern Europe (10 and 8 accessions, respectively). In accessions from East Asia, three cultivars were with the homozygote *S* allele ('Kheikhek14', 'Dong doe 641' and 'Ken feng 20', China), and one was heterozygous ('Kharbin', China). In accessions from Northern America, two cultivars were with the homozygous *S* allele ('KG 20', Canada and 'Carola', USA), and three were heterozygous genotypes ('Maple Arrow' and 'GEO', Canada and 'Linkoln', USA). In accessions from Western Europe, one cultivar carried the *S* allele ('Sepia', France), and one was heterozygous ('Fiskeby5', Sweden). All Central Asian accessions carried the homozygous *R* allele of the *pdh1* (Fig. 4).

The results of field screening for PD of the average data for the two years of experiments and genotyping data by

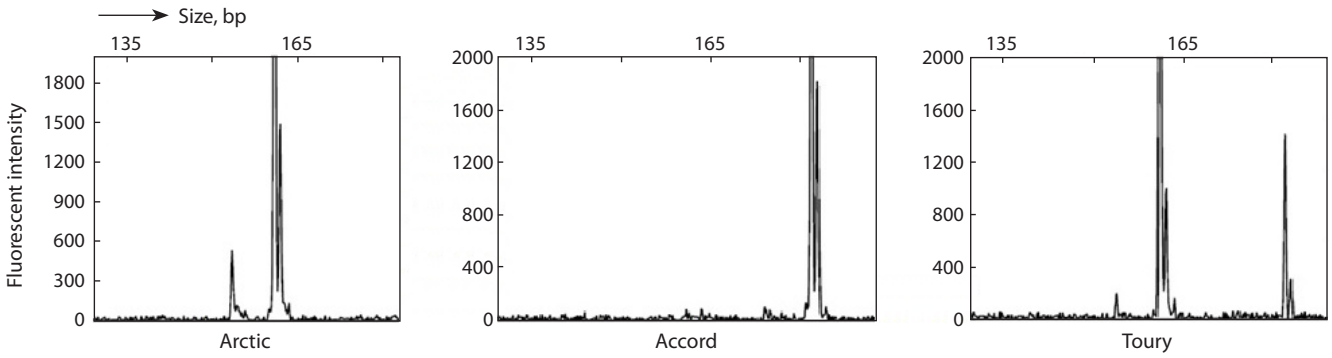

Fig. 3. Amplification products of specific SNP marker for the *Pdh1* gene in Arctic, Accord, and Toury soybean varieties with *S* and *R* alleles and heterozygote (*H*), respectively, detected by Genetic Analyzer 3500.

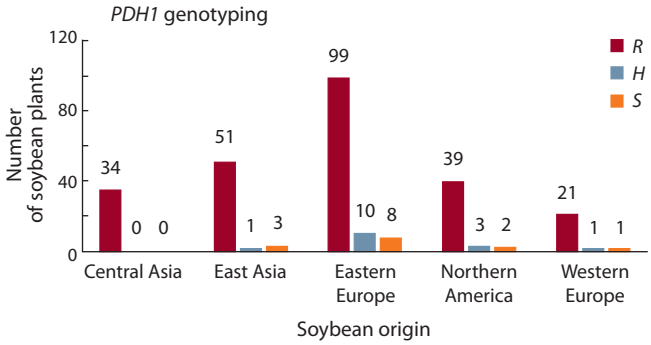

Fig. 4. The genotyping results of the soybean collection studied using an allele-specific SNP marker of the *Pdh1* gene.

S – homozygous genotypes with the susceptible allele, *R* – homozygous genotypes with the resistant allele, *H* – heterozygotes.

DNA marker showed a moderate correlation link ($p < 0.01$). Comparative assessment of PD in field studies and *Pdh1* genotyping indicated that in 14 accessions with the homozygous *S* allele, only seven cultivars were susceptible to PD in both years, and ten samples, in one of the two studies years (Supplementary Material 1). These seven cultivars were from Eastern Europe (6 accessions) and Northern America (1 accession). In 244 identified samples with the homozygous *R* allele, four accessions were susceptible to PD in both years, and 19 accessions, in at least one out of two studied years (Supplementary Material 1). These four cultivars were from Eastern Europe (3) and North America (1).

Linkage disequilibrium, population structure, and genome-wide association study

After filtering the genotyping data by MAFs, missing data in individuals, and heterozygous calls, a total of 4,651 SNPs remained. The average density of the SNP map was one marker per 246 Kb. Linkage disequilibrium (LD) decayed at 3.3 Mb for the whole genome at R2 of 0.1 (Fig. 5*a*). The population structure (Q) based on the results of STRUCTURE and STRUCTURE Harvester analyses showed three subpopulations (Fig. 5*c*). The Q matrix was developed using $K = 3$ as the optimum (Fig. 5*b*).

The Manhattan plot with SNP markers associated with PD and the QQ plot are illustrated in Figure 6, the Manhattan plot and the QQ plot of each year of the experiment are illustrated in Supplementary Materials 3, 4. The threshold is 1.0×10^{-5} at a significance level of 5 % after Bonferroni multiple test correction. A significance threshold of 5 % FDR was used to identify putative SNP associations. If two SNPs were closer than the genome average LD decay value of 3.3 Mbp, they were considered to belong to the same locus.

The GWAS with significance thresholds of FDR and Bonferroni correction allowed the identification of three QTLs for PD on chromosomes 10, 13, and 16 (Fig. 6, Table 2, Supplementary Materials 3–5). For each identified QTL, one most significant SNP marker with the lowest *p*-value was selected: Gm10_47774781 on chromosome 10, Gm13_6207590 on chromosome 13, and Gm16_29681065 on chromosome 16. The information about the marker positions on the chromosomes, *p*-values, effects, and phenotypic variations for alleles is shown in Table 2.

Gm16_29681065 was located in the vicinity of *Pdh1* on chromosome 16 (Table 2). Other two minor QTLs were identified on chromosomes 10 and 13. Identified SNPs with the most significant *p*-values of Gm16_29681065, Gm10_47774781, and Gm13_6207590 were designated as *qPD16-1*, *qPD10-1*, and *qPD13-1*.

The influence of the allelic status of the most significant SNPs of three stable QTLs for the PD phenotype is shown in Table 3. The results in Table 3 indicate that the combination of effective SNP alleles (TTG) in three QTLs resulted in PD resistance with a value of 0.1. In contrast, the combination of alternative alleles (GCA) showed susceptibility to PD with a value of 3.9. Interestingly, two plants with the TCA combination (a resistant allele for Gm16_29681065 and two susceptible alleles for Gm10_47774781 and Gm13_6207590) showed PD phenotype with the value of 4.5 (Table 3), suggesting that the effective allele in Gm16_29681065 alone is not sufficient for PD resistance.

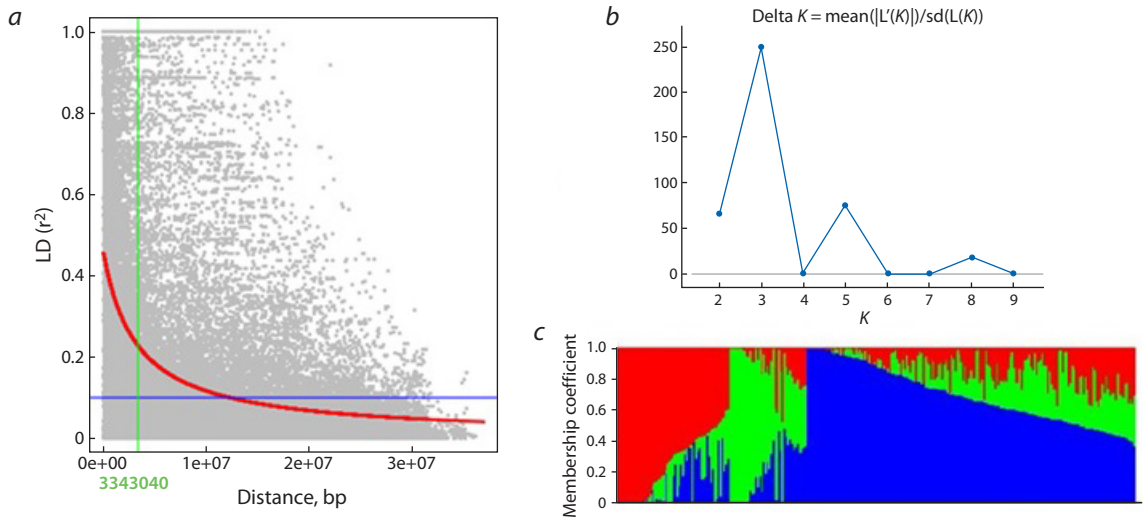

Fig. 5. *a*, LD decay plot of 4,651 SNPs through the whole soybean genome; *b*, Delta *K* for differing numbers of subpopulations; *c*, bar plot of estimated population structure of 273 soybean genotypes on $K = 3$.

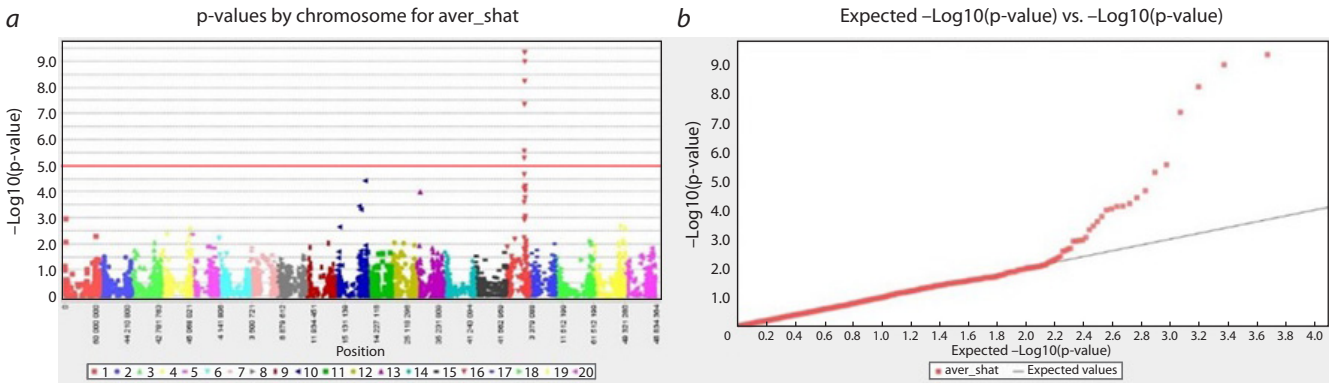

Fig. 6. Manhattan (*a*) and QQ plots (*b*) for the pod dehiscence trait in the world soybean collection for average data of 2019 and 2021 in the Almaty region.

Table 2. The list of identified significant SNP markers associated with PD for 2019 and 2021 and the average data for the two years of the experiment using the genome-wide association study

Parameter	Gm16_29681065, *qPD16-1*	Gm10_47774781, *qPD10-1*	Gm13_6207590, *qPD13-1*
Chromosome	16	10	13
Position, bp	29681065	47774781	6207590
Allele	G	C	A
		2019	
p-value/FDR	1.7576E-10/8,17E-07	0.00203/4,50E-01	0.00391/6,99E-01
Effect*	1.05999	0.36111	0.29456
R2**	0.1645	0.03655	0.03527
		2021	
p-value/FDR	1.5159E-6/2,35E-03	2.1195E-5/1,97E-02	5.89E-4/1,61E-01
Allele effect	0.38581	0.24986	0.17689
R2	0.08677	0.06757	0.04363
		Average	
p-value/FDR	4.7063E-10/2,19E-06	3.8127E-5/2,22E-02	1.0165E-4/3,64E-02
Allele effect	0.45428	0.20783	0.1743
R2	0.15097	0.06413	0.05917
Candidate loci	*Pdh1*/Glyma16g25580 (Gm16:29601346...29601897) (Funatsuki et al., 2014)	Glyma10g40330 (Calmodulin-binding protein, start 47773565–stop 47775599) (Schmutz et al., 2010)	Near Glyma13g05890 (Calmodulin-binding protein, start 6199393–stop 6203098) (Schmutz et al., 2010)

* Absolute effect; ** R2 – marker phenotypic variation.

Table 3. Mean of PD scores for allelic combinations of SNP markers in three identified quantitative trait loci of PD in field conditions

Gm16_29681065	Gm10_47774781	Gm13_6207590	Number of lines	Mean PD score
T	T	G	192	0.1
T	T	A	29	0.3
T	C	G	19	0.4
T	C	A	2	4.5
G	T	G	9	1.5
G	C	G	4	4.5
G	C	A	4	3.9

Discussion

The assessment of the collection in the field conditions of the southeast of Kazakhstan has confirmed a high negative impact of PD on yield performance (Fig. 2). The field evaluation of average data revealed that 32 genotypes were susceptible to PD in at least one of the two studied years (Fig. 1). The phenotypic results for PD over two years of study were stable and largely coincided with genotypic results using an allele-specific SNP marker of *Pdh1*, confirming the fact that *Pdh1* played a critical role in soybean expansion (Funatsuki et al., 2014). Nevertheless, 19 out of 244 accessions with homozygous *R* alleles showed susceptibility to PD in the field conditions of southeast Kazakhstan, suggesting that more genes are involved in regulating PD. Therefore, GWAS was applied to identify additional genetic factors that can potentially be involved in the genetic control of PD. The application of GWAS suggested that three stable QTLs for PD were significant in this study.

The three identified QTLs (*qPD10-1*, *qPD13-1*, and *qPD16-1*) were located on chromosomes 10, 13, and 16, respectively (Table 2). As QTL *qPD16-1* was highly significant both in 2019 and 2021, it can be considered a major genetic factor showing a remarkable effect on PD. The location of QTN *qPD16-1* coincided with the genetic position of *Pdh1* (Funatsuki et al., 2014) (Table 2). The literature survey suggests that *Pdh1* (Gm16:29601346–Gm16:29601897) encodes a dirigent family protein known to be involved in lignification, which increases dehiscing forces by promoting torsion of dried pod walls (Funatsuki et al., 2014). The loss-of-function *pdh1* gene has been widely used in soybean breeding as a pod dehiscence resistance gene (Funatsuki et al., 2014).

The other significant SNP for PD identified on chromosome 10, *qPD10-1*, was located in Glyma10g40330 (Schmutz et al., 2010), the gene that is responsible for the expression of plant calmodulin-binding protein (soybase.org). Previously, another QTL for PD was identified on chromosome 10, which was located within 10 cM of Satt243 (Gm10:46088332–46088382, soybase.org) (Kang et al., 2009), suggesting a strong genetic linkage between QTNs in two association findings. Interestingly, the significant QTL identified on chromosome 13 was located in the vicinity of Glyma13g05890, which is also expressing plant calmodulin-binding protein (Schmutz et al., 2010; soybase.org).

The results of influences of all three identified genetic factors on PD performance suggest that although the role of *qPD16-1* is remarkable, the allelic statuses of Gm10_47774781 and Gm13_6207590 are also essential (Table 3). Hence, it can be hypothesized that calmodulin-binding protein is part of the gene network controlling PD. Calmodulin (CAM) is a Ca^{2+} sensor known to regulate the activity of many eucaryote proteins and plays an important role in plant growth and development (Yu et al., 2021). An increasing number of studies have illustrated that plant calcium signals play a vital role in life processes by acting as a messenger transducer in the complicated signal network to regulate plant growth and development and the response and adaptation to environmental stresses (Hong-Bo et al., 2008). Hypothetically, drought or high temperature as environmental stress can induce responses by activating calmodulin-binding protein, leading to a change in the structure of soybean pods. In general, the results of the soybean PD study in conditions of southeast Kazakhstan suggest that it is controlled by one major and two minor QTLs, which is congruent with results of previous reports, where one major and few minor QTLs were revealed (Tsuchiya, 1987; Bailey et al., 1997; Ogutcen et al., 2018; Seo et al., 2020). Nevertheless, *qPD13-1*, identified in this work, has not been reported in any previous PD studies, and, therefore, it can be considered a putatively novel genetic factor for the regulation of PD in soybeans.

Conclusion

The evaluation of the collection consisting of 273 soybean accessions with different origins for PD has confirmed a strong influence of the *Pdh1* gene on trait performance and a negative impact on yield and yield components over two studied seasons in southeast Kazakhstan. The application of GWAS has allowed the identification of one major (*qPD16-1*) and two minor (*qPD10-1* and *qPD13-1*) QTLs for PD. The location of the major QTL has coincided with the physical position of the *Pdh1*. Two minor QTLs have been associated with the genes for calmodulin-binding protein on chromosomes 10 and 13. The assessment of available scientific reports for the genetic control of PD suggests that the QTL for PD on chromosome 13 is a novel genetic factor for regulating the studied trait.

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Prospects for mineral biofortification of wheat: classical breeding and agronomy

I.N. Leonova [1], E.V. Ageeva [2], V.K. Shumny[1]

[1] Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
[2] Siberian Research Institute of Plant Production and Breeding – Branch of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Krasnoobsk, Novosibirsk region, Russia

leonova@bionet.nsc.ru

Abstract. Low intake of micro- and macroelements and vitamins in food negatively affects the health of more than two billion people around the world provoking chronic diseases. For the majority of the world's population, these are soft and durum wheats that provide beneficial nutrients, however their modern high-yielding varieties have a significantly depleted grain mineral composition that have reduced mineral intake through food. Biofortification is a new research trend, whose main goal is to improve the nutritional qualities of agricultural crops using a set of classical (hybridization and selection) methods as well and the modern ones employing gene/QTL mapping, bioinformatic analysis, transgenesis, mutagenesis and genome editing. Using the classical breeding methods, biofortified varieties have been bred as a part of various international programs funded by HarvestPlus, CIMMYT, ICARDA. Despite the promise of transgenesis and genome editing, these labor-intensive methods require significant investments, so these technologies, when applied to wheat, are still at the development stage and cannot be applied routinely. In recent years, the interest in wheat biofortification has increased due to the advances in mapping genes and QTLs for agronomically important traits. The new markers obtained from wheat genome sequencing and application of bioinformatic methods (GWAS, meta-QTL analysis) has expanded our knowledge on the traits that determine the grain mineral concentration and has identified the key gene candidates. This review describes the current research on genetic biofortification of wheat in the world and in Russia and provides information on the use of cultivated and wild-relative germplasms to expand the genetic diversity of modern wheat varieties.

Key words: wheat; microelements; macroelements; breeding; agronomy; biofortification.

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Перспективы биообогащения пшеницы минералами: классическая селекция и агрономия

И.Н. Леонова [1], Е.В. Агеева [2], В.К. Шумный[1]

[1] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Сибирский научно-исследовательский институт растениеводства и селекции – филиал Федерального исследовательского центра Институт цитологии и генетики Сибирского отделения Российской академии наук, р.п. Краснообск, Новосибирская область, Россия

leonova@bionet.nsc.ru

Аннотация. Недостаток потребления микро- и макроэлементов и витаминов в продуктах питания, который затрагивает более двух миллиардов человек на земном шаре, негативно сказывается на здоровье и приводит к развитию хронических заболеваний. Одним из источников полезных нутриентов является пшеница, которая обеспечивает пищевой энергией большинство населения мира. Создание современных высокоурожайных сортов привело к значительному обеднению минерального состава зерна и сокращению потребления минералов через продукты питания. Биофортификация – активно развивающееся направление, основной целью которого является улучшение питательных качеств сельскохозяйственных культур с помощью комплекса классических и современных методов. К числу основных технологий, используемых в программах биофортификации пшеницы, можно отнести традиционную селекцию, включающую методы гибридизации и отбора, современную селекцию с дополнительным привлечением методов картирования генов/QTL и биоинформатического анализа, трансгенез, мутагенез и геномное редактирование. Успехи в создании биообогащенных

сортов были достигнуты в рамках различных международных программ, финансируемых HarvestPlus, CIMMYT, ICARDA, с помощью традиционной селекции и агрономических методов. Несмотря на перспективность методов трансгенеза и геномного редактирования для создания биообогащенных культур, они требуют значительных инвестиционных вложений и трудозатратны, поэтому данные технологии применительно к пшенице находятся в стадии разработки и не имеют пока практического выхода. В последние годы интерес к биообогащению пшеницы возрос в связи с успехами в области картирования генов и QTL для хозяйственно важных признаков. Разработка новых маркеров на основе результатов секвенирования генома пшеницы и привлечение биоинформатических методов анализа (GWAS, meta-QTL) расширили информацию по контролю признаков, определяющих содержание минералов в зерне, и выявили ключевые гены-кандидаты. В данном обзоре описано современное состояние исследований в области генетической биофортификации пшеницы в мире и в России. Приведены сведения об использовании гермоплазмы культурных и дикорастущих родственников для расширения генетического разнообразия современных сортов пшеницы.
Ключевые слова: пшеница; микроэлементы; макроэлементы; селекция; агрономия; биофортификация.

Introduction

As a source of complete plant protein, minerals, micro- and macronutrients and vitamins, wheat plays an important role for the world's population. Consuming wheat products, the population obtains, on average, up to 20–30 % of calories per day; in some developing countries this figure is as high as 70 % (Shewry, 2009a; Shiferaw et al., 2013; Tadesse et al., 2019). To meet the growing demand for wheat grain, increasing yields has been the main focus for the breeders since the 1960s. Expansion of planted areas and introduction of new high-yielding varieties has gradually increased the world's wheat production, so, according to the FAO, the grain harvest was estimated at 805.6 million tons in 2023 compared to less than 600 million tons in 2000 (https://www.fao.org/worldfoodsituation/csdb/ru). Compared to then, significant yield increases of 1.3 to 1.8 times have been observed in major wheat-producing countries such as China, India, Russia and the United States (https://www.fao.org/faostat/ru/#country/).

However, this success in increasing wheat yields achieved through introduction of high-yielding varieties has been accompanied by deteriorated grain quality, reduced contents of protein, gluten and minerals that determine the nutritional value of the final product (Mitrofanova, Khakimova, 2017; Helguera et al., 2020). Published data indicate that the micro- and macronutrient contents in the grain of modern varieties have been significantly lower than those in ancient varieties and wild relatives (Salantur, Karaoğlu, 2021; Zeibig et al., 2022).

The micro- and macronutrients play an important role in many processes of plant development such as seed germination, root system development and yield formation (Marschner, 1995). They are also indispensable when it comes to photosynthesis and respiration and stress resistance regulation (De Santis et al., 2021; Shoormij et al., 2022; Khan et al., 2023). The list of macronutrients now considered essential for a healthy lifestyle and normal body function includes sodium (Na), potassium (K), magnesium (Mg), calcium (Ca), chlorine (Cl), phosphorus (P), and sulfur (S). Iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), iodine (I), and selenium (Se) are commonly recognized as indispensable microelements (Jomova et al., 2022; Ali A.A.H., 2023). Some nutritionists additionally include bromine (Br), vanadium (V), silicon (Si), nickel (Ni) and chromium (Cr) on that list, but the data on the positive effects of these elements in animals and humans are currently contradictory (Vincent, 2017; Genchi et al., 2020).

Deficiencies in micronutrient intake with food, or the so-called "hidden hunger", lead to the development of chronic diseases, reduced mental development and even increased mortality in most developing countries (Faber et al., 2014; Lockyer et al., 2018). Deficiency in Na, K, Ca, Mg, P results in nervous, cardiovascular, skeletal and muscular systems impairments. Among the essentials, these are the deficiencies of Fe, Zn, I and Se that are of particular concern, since they are involved in hemoglobin synthesis; regulation of the functions of a number of enzymes, including insulin; metabolism; cancer-cell suppression, etc. (Prashanth et al., 2015; Islam et al., 2023).

Currently, the improvement of wheat nutritional properties by increasing the concentration and bioavailability of essential micro- and macronutrients has become a priority in the field of wheat genetics and breeding. This direction commonly known as biofortification is developed through various approaches, the main ones being agronomic and genetic biofortification using both traditional breeding methods and modern molecular genetic approaches. The paper reviews the results obtained by the agronomic methods and classical breeding employing gene mapping technologies, and considers the prospects for their use in the development of biofortified wheat varieties.

Mineral composition of wheat and its wild relatives

Mineral composition in wheat whole grains and flour varies a lot to be determined by the genotype, environments, soil composition, presence of mineral fertilizers and other agronomic factors. A significant contribution to the phenotypic manifestation and inheritance of the trait is made by the genotype, because it allows one to use samples with increased mineral content to breed new lines of wheat.

The mineral content in the grain of modern bread wheat varieties may change considerably: such elements as Zn, Fe, Cu, Mn range up to 40, 50, 4 and 38 μg/g, respectively; while the content K, Mg and Ca does not exceed 4,200, 1,150 and 370 μg/g on average (Murphy et al., 2008; Zhao et al., 2009; Khokhar et al., 2018; Morgounov et al., 2022; Potapova et al., 2023). Although today's durum wheats do not differ signifi-

cantly from soft ones in the concentration of major mineral elements (Ficco et al., 2009; Shewry et al., 2023), some authors indicate that Zn and Fe content in the grain of many durum wheat varieties has been significantly higher (Cakmak et al., 2010; Rachoń et al., 2012).

Modern wheat varieties have lower concentrations of macro- and micronutrients compared to their ancient, and wild and cultivated relatives. A number of studies have shown that the decrease in micronutrient content is not always related to changes in climatic factors or soil characteristics (Garvin et al., 2006; Ficco et al., 2009). M.S. Fan et al. (2008) conducted an extensive study of soil composition and the changes in Zn, Fe, Cu and Mn contents in wheat grains over 160 years. The mineral content was shown to have remained stable from 1845 to the 1960s, but then it declined significantly due to the introduction of yielding dwarfing varieties. This trend was maintained regardless of changes in the concentration of soil elements or the administration of organic and inorganic fertilizers. In other words, the reduced-height (*Rht*) genes in durum and soft wheat are accompanied by reduced micronutrient concentrations, the reduction level varies and depends on the genetic background of a variety (Velu et al., 2017a). Some authors note there are negative correlations between the yield of modern varieties and their Zn and Fe content. This may be the reason for the decrease in the concentration of the minerals in grain due to the cultivation of highly productive varieties (Monasterio, Graham, 2000; Garvin et al., 2006).

Breeding a biofortified wheat variety causes a problem of maintaining a high mineral content in the final products, since a significant part of micronutrients is concentrated in the grain shell, e. g., Zn, Fe and Cu concentrations reduce 2 to 10 times in flour if compared to that in whole grain, while in the bran made of grain hulls it remains several times higher (Peterson et al., 1986; Ciudad-Mulero et al., 2021). A good alternative may be using whole-wheat flour or adding bran that contains much more essentials into flour. Different proportions of wheat bran added into flour increase the Fe content in baked products, the greatest effect observed when adding 10 % of bran, which makes the bread comparable to that made of whole-wheat flour (Butt et al., 2004).

Screening the germplasms of wild and cultivated wheat relatives has also revealed significant differences in mineral concentrations. Despite the wide variability in Ca, Mg, K, Zn, Fe, Mn and Cu contents in the diploid and tetraploid ancestors of *T. durum*, *T. dicoccum*, *T. monococcum*, *T. araraticum*, *T. timopheevii*, and *Ae. tauschii*, scientists have observed that hexaploid wheat *T. aestivum*, on average, is inferior to them in the concentration of most elements (Marschner, 1995; Cakmak et al., 2004; Gupta P.K. et al., 2021; Zeibig et al., 2024). Zn and Fe concentrations in the grain of various representatives of the genus *Aegilops* (*Ae. searsii*, *Ae. umbellulata*, *Ae. caudata*, *Ae. geniculata*, etc.) are 2–3 times higher than those in modern hexaploid wheat cultivars (Gupta P.K. et al., 2021; Zeibig et al., 2022). High genetic diversity in relation to the mineral composition was found in wild spelt *T. dicoccoides*; a combination of high zinc, iron and protein contents in grain and high yield was also observed for some spelt varieties (Peleg et al., 2008; Chatzav et al., 2010).

Significant intra-population diversity is the basis for utilizing the genetic potential of wild and cultivated relatives as a source of high mineral content in grain for the presence of positive correlations between some element concentrations (Zn, Fe, Mg), protein content and yield allows for simultaneous improvement of several quality parameters without reducing productivity (Oury et al., 2006; Chatzav et al., 2010).

To improve the mineral composition, various introgression, addition, and substitution lines derived from hybridization of modern soft and durum wheat varieties with wild and cultivated relatives have been developed (Wang S. et al., 2011; Farkas et al., 2014; Savin et al., 2018). Examination of the given resources has enabled for identification of the accessions with better characteristics than the original commercial wheat varieties. It has also made it possible to detect the critical chromosomes containing targeted genetic factors to establish a basis for subsequent gene mapping.

An extensive source of genetic diversity for mineral composition in wheat are the synthetic hexaploid lines derived from the hybridization of different accessions of *T. turgidum* ssp. *durum* and *Ae. tauschii* (Alvarez, Guzmán, 2018; Morgounov et al., 2022). Using these synthetic lines, a large number of favorable target-gene alleles have been mapped that can be employed for genetic biofortification (Bhatta et al., 2018; Morgounov et al., 2022). However, a detailed analysis of the productivity of the accessions bred with these wheat relatives has shown that most of them are characterized by a decrease in yield and its components, depending on the genetic background of the recipient variety and the amount of alien genetic material (Calderini, Ortiz-Monasterio, 2003; Velu et al., 2017b), which significantly complicates the transfer of target genes into commercial wheat varieties.

Genetic biofortification

Conventional breeding is the most common and cost-effective biofortification method to improve the mineral composition of wheat grain. In this classical method, donors of high nutrient content are crossed with a recipient variety possessing necessary economically important traits to select the sought trait in subsequent generations. If a foreign species is used as a donor, the process may be followed by several backcrossing cycles to transfer the targeted introgressed fragment and reduce the amount of foreign genetic material.

As a part of biofortification programs carried out in the major international centers involved in the study of cereal crops (CGIAR, CIMMYT, HarvestPlus, ICARDA), the results of screening of their collections of modern wheat varieties, landraces and wild species have been used to determine the mineral composition variability, develop recommendations and create pre-breeding lines. (Monasterio, Graham, 2000; Peleg et al., 2008; Ficco et al., 2009).

The use of traditional breeding methods for biofortification of wheat grain became a topical issue in Europe after the HEALTHGRAIN program was initiated (2005–2010) to

summon 43 partners from 17 countries participated. Thanks to this program modern varieties and breeding lines, landraces of bread wheat and other cereal crops (rye, barley, oats) from European countries were evaluated for phytochemical components and mineral composition at several experimental plots. The results have shown that a large part of the trait variations was genetically determined, so the selected material may be available for breeding programs (Shewry, 2009b; Van Der Kamp et al., 2014).

Since 2003, HarvestPlus program has been investing heavily to develop biofortified varieties of wheat, rice, corn, millet, beans, sweet potato and other crops with higher levels of vitamin A, Fe and Zn. Their wheat biofortification program is underway in Africa (Egypt, Ethiopia, Madagascar, Nigeria, South Africa, Zambia and Zimbabwe), Asia (Afghanistan, Bangladesh, China, India, Nepal, Pakistan, the Philippines) and Latin America (Bolivia, Brazil, Mexico) (https://www.harvestplus.org/biofortification-hub). To date, under the program, 37 biofortified wheat varieties for countries in Asia and Africa have been developed, of which 12 are high-yielding and resistant to fungal diseases (Andersson et al., 2017; Bouis, Saltzman, 2017; Kamble et al., 2022). The study of Zn-biofortified varieties developed in India under the HarvestPlus brought the authors of the experiment to a conclusion that despite the low contribution of genotype to the overall variability of Zn concentration in grain, the biofortified genotypes exhibited environmental stability when grown in different soil types, including those with low Zn content (Khokhar et al., 2018). A list of biofortified soft and durum wheats developed by major breeding institutions in India, Pakistan, Bangladesh, Nepal and Bolivia in collaboration with CIMMYT and recommended for commercial cultivation is presented in Gupta O.P. et al. (2022). These include durum wheat varieties HI8777 and MACS 4028 with Fe content of 48.7 and 46.1 mg/kg and Zn content of 40.3 and 43.6 mg/kg, respectively; and soft wheat varieties WB 02, HI 1633, DBW 187, DBW 332 and PBW 757, whose concentration of these elements exceeds 40 mg/kg.

In recent years, close attention has been paid to the development of biofortified wheat genotypes of non-standard grain color (blue, purple, black) that differ from conventional red-grain and white-grain varieties by a high content of anthocyanins having antioxidant, antimicrobial and anticarcinogenic activity. Investigation of the pigmented samples has shown that some of them have additional characteristics such as increased protein and micro- and macronutrient content (Sharma S. et al., 2018; Xia et al., 2020; Dhua et al., 2021; Liu Y. et al., 2021). Analysis of the flour made of blue, green and black grains has found that the pigmented varieties exceed the standard ones in protein and amino acid content by 7–18 %, while their zinc content is almost 2-fold higher, and that of Fe and Mn varies from 8 to 40 % (Tian et al., 2018). There is also evidence that the blue-grain wheat has higher iron and zinc contents if compared to those of purple, red and white varieties (Ficco et al., 2014). Experiments to search for samples with high Se content that has antitumor activity have been conducted for the pigmented wheats (Xia et al., 2020). They demonstrated that when the plants were sprayed with Se or when the last was applied to soil, the purple-grain varieties accumulated more Se in grain if compared to the white-grain ones. However, according to other authors, in the absence of additional selenium treatments, the blue- and purple-grain varieties were inferior to standard wheat varieties by almost 5 times (Phuong et al., 2017).

Pigmented wheats may contain increased amounts of gluten, anthocyanin and minerals, and for that reason they are considered as a promising source of useful nutrients for bakery and pasta products. However, the studies having investigated the detailed qualitative characteristics of the final products made of pigmented-wheat flour are few and include mainly the assessment of anthocyanins, protein and gluten content, dough characterization and a description of organoleptic properties (Pasqualone et al., 2015; Vasilova et al., 2021; Sharma N. et al., 2022; Fitileva, Sibikeev, 2023; Gordeeva et al., 2023). Nevertheless, encouraging results proving that such products retain significantly more beneficial nutrients while processing have already been obtained (Padhy et al., 2022). For example, A. Kumari et al. (2020) analyzing the chapati baked from pigmented wheat varieties showed that the wheat samples ranged in the following descending order in terms of their phenolic content, anthocyanins and antioxidant activity: black > blue > purple > white grain. Currently, colored-grain varieties are considered as a promising source of bioactive substances and high antioxidant activity.

Quantitative trait loci mapping

An important stage of biofortification is the selection of potential genotypes containing target loci, whose presence leads to an increase in mineral elements in grain. Currently, it is DNA markers being used for nearly three decades for mapping quantitative trait loci (QTL) and for marker-assisted and genomic selection (Collard, Mackill, 2008). That is of great importance, since a detected QTL localization and position on a chromosome allows one to understand the genetic basis of a trait, identify the loci controlling mineral-elements content as well as new QTLs and, based on the information obtained, select the genotypes suitable for breeding.

Two approaches are used to localize target loci and identify new gene alleles: genetic mapping on the populations raised from biparental crosses, and genome-wide association study (GWAS), whose main advantage is the use of the genotype panels characterized by high genetic diversity (Collard, Mackill, 2008; Tibbs Cortes et al., 2021).

In the last 15 years, a sufficient number of papers have been published on mapping of the QTLs whose presence determines essentials content in wheat grain. It should be noted that most of these studies have been conducted mainly to identify genomic regions controlling Zn and Fe concentrations, as these elements are considered indispensable for human health (Peleg et al., 2009; Tiwari et al., 2009; Wang S. et al., 2011; Hao et al., 2014; Pu et al., 2014). GWAS has enabled for more accurate mapping of the genomic loci, so new previously unpublished QTLs have been identified, and functional candidate genes have been searched for in the regions of target loci (Bhatta et al., 2018; Alomari et al., 2019; Rathan et al., 2022; Tadesse et al., 2023). Comprehensive

research to identify the key genomic regions for Zn and Fe biofortification in soft wheat was conducted by P. Juliana et al. (2022), who, using a panel of 5,585 advanced-generation pre-breeding lines, identified 141 markers on all wheat chromosomes except for 3A and 7D. The results summarizing the QTL localizations for Zn and Fe contents in wheat grain are presented in part in review articles (Garcia-Oliveira et al., 2018; Gupta O.P. et al., 2022). Currently, researchers are accumulating information on the most informative loci, their localization and validating SNP-KASP markers. So far, only the first steps have been taken towards developing the KASP markers based on the mapped QTLs (Wang Y. et al., 2021; Sun M. et al., 2023) and there is no available information on their specificity and practical use.

Only a limited number of studies cover the issue of genetic and association mapping of the QTLs responsible for other mineral elements (Alomari et al., 2017; Manickavelu et al., 2017; Wang P. et al., 2017; Qiao et al., 2021; Hao et al., 2024). Comparative genomic and meta-QTL analyses identified more than 400 stable loci for some of which pleiotropic effects were shown in relation to different mineral elements and yield components (Shariatipour et al., 2021; Singh et al., 2022; Potapova et al., 2023; Cabas-Lühmann et al., 2024). A GWAS conducted for 205 winter soft wheat genotypes from China revealed more than 280 marker-trait associations with Ca, Mn, Cu, and Se contents in different wheat chromosomes. The study also demonstrated that the gene clusters in chromosomes 3B and 5A (for Ca), 4B (for Cu), and 1B (for Mn) had the highest contribution to their content (Wang W. et al., 2021). Based on a whole-genome analysis of 252 soft wheat cultivars for Se content, it was concluded that the use of the SNP markers linked to target loci in chromosomes 5D and 1D could increase Se concentration by 6.62 % during genomic selection (Tadesse et al., 2023). A GWAS performed on a panel of 768 cultivars found the genomic regions associated with Cu, Fe, K, Mg, Mn, P, Se, and Zn concentrations in soft wheat and the stably expressed candidate genes located in the QTL localization regions (Hao et al., 2024). Eleven loci associated with calcium accumulation were detected in chromosomes 2A, 3A (2 loci), 3B (2 loci), 3D, 4A, 4B, 5B (2 loci), and 6A, of which four QTLs were stably expressed under different environmental conditions (Shi X. et al., 2022). Candidate-gene study by these authors identified the *TraesCS4A02G428900* gene in chromosome 4A, whose high expression may be associated with calcium accumulation in wheat grains.

To find sources and donors of the efficient loci associated with high mineral concentrations in grain, a search for new loci was conducted using various bread wheat relatives and synthetic hexaploid wheats (SHWs) (see the Table). The SHWs obtained from crosses between tetraploid species (*T. durum*, *T. dicoccum*) and diploid *Ae. tauschii* have become a source of new gene alleles for various agronomically important traits. According to Z.E. Pu et al. (2014), 22 of the 29 alleles responsible for increased concentration of Zn, Fe, Mn, Cu and Se in the grain of recombinant inbred lines originate from the genome of a synthetic line derived from the crossing of *T. turgidum* ssp. *turgidum* and *Ae. tauschii* ssp. *tauschii*. A significant number of loci, including novel ones, have been identified in the genome-D chromosomes originating from different varieties of *Ae. tauschii*, which demonstrates the high potential of this species in increasing the content of such elements as Ca, Co, Cu, Li, Mg, Mn and Ni in grain (Bhatta et al., 2018; Krishnappa et al., 2021; Morgounov et al., 2022).

A number of studies have shown that the presence of foreign chromosomes in the genomes of substitution, introgression and addition wheat lines leads to increased concentrations of Zn, Fe, and other minerals (Wang S. et al., 2011; Velu et al., 2017c; Gupta P.K. et al., 2020; Potapova et al., 2023). In diploid wheat species (*T. monococcum*, *T. boeoticum*), two loci responsible for Fe content were identified in chromosomes 2A and 7A and one responsible for Zn in chromosome 7A (Tiwari et al., 2009; see the Table). In different populations of cultivated and wild tetraploid species, recombinant inbred and synthetic lines have had many QTLs originating from the A and B genomes of *T. durum*, *T. dicoccum*, and *T. dicoccoides* and associated with Fe and Zn content (Peleg et al., 2009; Crespo-Herrera et al., 2016, 2017; Cabas-Lühmann et al., 2024). It is noteworthy that a number of mapped QTLs for Zn, Fe, Mn, and other minerals have no negative effects on grain protein content, 1,000-grain weight, and yield in general, which allows one to improve these varieties for several traits simultaneously (Uauy et al., 2006; Liu J. et al., 2021; Cabas-Lühmann et al., 2024). Also, many studies have shown a high level of heritability of the studied traits that indicates the genotype's significant contribution. It will make it possible to use the samples with foreign translocations as a source of target genetic factors while breeding (see the Table).

Agronomic methods

The simplest and most accessible of all biofortification methods is employment of the fertilizers enriched with micro- and macroelements that either applied to the soil or as a foliar treatment. Several studies suggest that applying different concentrations of nitrogen fertilizer alone or in combination with mineral supplements can have a positive effect on grain micronutrient content (Shi R. et al., 2010; Kutman et al., 2011; Niyigaba et al., 2019). As for the effectiveness of different methods (seed treatment, soil fertilization, and foliar spraying) in terms of their effects on the yield, protein content, and mineral concentration, foliar spraying has so far been regarded as the most effective one (Stepien, Wojtkowiak, 2016; Hassan et al., 2019; Saquee et al., 2023), e. g., the efficacy analysis of foliar spraying of wheat with Zn fertilizers conducted on 23 experimental fields in seven countries (China, India, Kazakhstan, Mexico, Pakistan, Turkey, and Zambia) showed an 80–90 % increase of Zn concentration in grain and no reduction in yield (Zou et al., 2012).

Many authors consider Zn solutions for foliage fertilizing an important tool for ensuring proper zinc concentrations in vegetative tissues during grain filling that increases Zn concentration in grain (Cakmak et al., 2010; Velu et al., 2014). The efficacy of such a treatment was demonstrated in experiments on Se biofortification of durum wheat (De Vita

Chromosomal localization of the loci associated with grain mineral content in synthetic hexaploid lines and wheat relatives that were detected using genetic mapping and GWAS

Mineral	Mapping population/ Methodology	Chromosome	Concentration*, mg/kg	Heritability (h^2)	References
Zn	RIL (*T. durum/Ae. tauschii* × *T. aestivum*)/ QTL mapping	2D, 3D, 4D, 5B	43.9	NA**	Pu et al., 2014
	SHW (*T. durum* × *Ae. tauschii*)/GWAS	1A, 2A, 3A, 3B, 4A, 4B, 5A, 6B	23.1	0.65	Bhatta et al., 2018
	T. boeoticum × *T. monococcum*/ QTL mapping	7A	32.4	NA	Tiwari et al., 2009
	T. aestivum × *T. dicoccoides*/GWAS	1A, 2A	60.6	0.97	Liu J. et al., 2021
	RIL (*T. durum* × *T. dicoccoides*)/ QTL mapping	2A, 5A, 6B, 7A, 7B	58.0	0.62	Peleg et al., 2009
	RIL (*T. dicoccum/Ae. tauschii* × *T. aestivum*)/ QTL mapping	4B, 5A, 5B, 6B, 6D	54.9	0.79	Crespo-Herrera et al., 2016
	RIL (*T. spelta* × *T. aestivum*)/QTL mapping	2A, 2B, 3D, 6A, 6B	42.2	0.80	Srinivasa et al., 2014
	RIL (SHW × *T. spelta*)/QTL mapping	1A, 1B, 3B, 3D, 4A, 5B, 6A, 7B, 7D	57.2	0.65	Crespo-Herrera et al., 2017
	RIL (*T. durum* × *T. dicoccum*)/ QTL mapping	1B, 5A, 6A, 6B	60.2	0.73	Velu et al., 2017c
	Wheat-*Aegilops* substitution and additional lines/GWAS	1B, 2B, 3A, 3B, 5D, 6A, 6D, 7B	42.0	0.61	Kaur et al., 2023
	RIL (*T. aestivum* × *T. dicoccum/Ae. tauschii*)/ GWAS	2A, 2D, 7D	38.0	0.77	Krishnappa et al., 2021
	SHW (*T. durum* × *Ae. tauschii*)/GWAS	1B, 2B, 2D, 3D	47.4	0.44	Morgounov et al., 2022
Fe	RIL (*T. aestivum* × *T. dicoccum/Ae. tauschii*)/ GWAS	1D, 2A, 3B, 6D, 7D	37.0	0.81	Krishnappa et al., 2021
	Wheat-*Aegilops* substitution and additional lines/GWAS	1B, 2A, 2B, 3B, 3D, 5A, 5B, 5D, 6A, 6B, 6D, 7A, 7D	39.0	0.68	Kaur et al., 2023
	RIL (*T. durum* × *T. dicoccum*)/QTL mapping	1B, 3A, 3B, 5B	57.2	0.30	Velu et al., 2017c
	RIL (SHW × *T. spelta*)/QTL mapping	2A, 2B, 3A, 3B, 4A, 4B, 4D, 5B	34.3	0.35	Crespo-Herrera et al., 2017
	RIL (*T. durum/Ae. tauschii* × *T. aestivum*)/ QTL mapping	2B, 5B, 5D, 7D	72.6	NA	Pu et al., 2014
	RIL (*T. dicoccum/Ae. tauschii* × *T. aestivum*)/ QTL mapping	2B, 2D, 4B, 5A, 5B, 6A, 6B, 6D, 7D	37.3	0.62	Crespo-Herrera et al., 2016
	SHW (*T. durum* × *Ae. tauschii*)/GWAS	1A, 3A	39.4	0.78	Bhatta et al., 2018
	T. boeoticum × *T. monococcum*/ QTL mapping	2A, 7A	31.6	NA	Tiwari et al., 2009
	RIL (*T. durum* × *T. dicoccoides*)/ QTL mapping	2A, 2B, 3A, 3B, 4B, 5A, 6A, 6B, 7A, 7B	33.8	0.69	Peleg et al., 2009
	T. aestivum × *T. dicoccoides*/GWAS	3B, 4A, 4B, 5A, 7B	98.3	0.97	Liu J. et al., 2021
	RIL (*T. spelta* × *T. aestivum*)/QTL mapping	1A, 2A, 3B	41.1	0.79	Srinivasa et al., 2014
	SHW (*T. durum* × *Ae. tauschii*)/GWAS	4A, 7B	35.9	0.38	Morgounov et al., 2022
Cu	RIL (*T. durum/Ae. tauschii* × *T. aestivum*)/ QTL mapping	2A, 3D, 4A, 4D, 5A, 6D, 7B	5.86	NA	Pu et al., 2014
	SHW (*T. durum* × *Ae. tauschii*)/GWAS	1B, 2A, 3A, 3B, 4B, 5A, 5B, 5D, 6A, 6B	6.6	0.63	Bhatta et al., 2018

Table (end)

Mineral	Mapping population/ Methodology	Chromosome	Concentration*, mg/kg	Heritability (h^2)	References
Cu	RIL (*T. durum* × *T. dicoccoides)*/ QTL mapping	1A, 2A, 3B, 4A, 4B, 5A, 6A, 6B, 7A, 7B	6.9	0.76	Peleg et al., 2009
	SHW (*T. durum* × *Ae. tauschii*)/GWAS	2B, 6D	4.25	0.40	Morgounov et al., 2022
Mn	RIL (*T. durum/Ae. tauschii* × *T. aestivum*)/ QTL mapping	1A, 2A, 2D, 4D, 5D	26.99	NA	Pu et al., 2014
	SHW (*T. durum* × *Ae. tauschii*)/GWAS	2D, 3A, 4B, 5D, 6B	43.1	0.67	Bhatta et al., 2018
	T. aestivum × *T. dicoccoides*/GWAS	1B	33.4	0.94	Liu J. et al., 2021
	RIL (*T. durum* × *T. dicoccoides)*/ QTL mapping	2B, 7B	41.6	0.41	Peleg et al., 2009
	SHW (*T. durum* × *Ae. tauschii*)/GWAS	2A, 3A, 4B, 7B	42.5	0.41	Morgounov et al., 2022
Ca	SHW (*T. durum* × *Ae. tauschii*)/GWAS	1B, 2B, 2D, 3A, 3B, 3D, 6A, 6B, 7A	73.7	0.41	Bhatta et al., 2018
	RIL (*T. durum* × *T. dicoccoides)*/ QTL mapping	1A, 2B, 4A, 4B, 5B, 6A, 6B, 7B	435.5	0.79	Peleg et al., 2009
	SHW (*T. durum* × *Ae. tauschii*)/GWAS	3B, 5A, 5D, 6D	389.5	0.50	Morgounov et al., 2022
Mg	SHW (*T. durum* × *Ae. tauschii*)/GWAS	1B, 1D, 2D, 3A, 3B, 4A, 4B, 4D, 5B, 5D, 7A	1424.5	0.62	Bhatta et al., 2018
	RIL (*T. durum* × *T. dicoccoides)*/ QTL mapping	1B, 2A, 3A, 5B, 6A, 6B, 7A, 7B	1534.5	0.74	Peleg et al., 2009
	SHW (*T. durum* × *Ae. tauschii*)/GWAS	1B, 2A, 4B, 5A, 5B, 6D, 7B	1203.5	0.59	Morgounov et al., 2022
K	RIL (*T. durum* × *T. dicoccoides)*/ QTL mapping	1A, 2A, 1A, 2B, 5B, 6A, 6B, 7B	4568.4	0.58	Peleg et al., 2009
	SHW (*T. durum* × *Ae. tauschii*)/GWAS	3A, 7A	3924.5	0.44	Morgounov et al., 2022
Cd	SHW (*T. durum* × *Ae. tauschii*)/GWAS	1A, 2A, 2D, 3A, 6D	0.07	0.28	Bhatta et al., 2018
	SHW (*T. durum* × *Ae. tauschii*)/GWAS	1A, 1B, 2A, 2B, 3D, 4A, 4D, 5D, 7A, 7B	0.033	0.44	Morgounov et al., 2022
Se	RIL (*T. durum/Ae. tauschii* × *T. aestivum*)/ QTL mapping	3D, 4A, 5B, 7D	0.55	NA	Pu et al., 2014

* Average values of field estimations; ** no data available (NA).

et al., 2017). According to the authors, Se concentration after grinding as well as in pasta increased by 11 %, while there was no decrease in other quality indicators, such as yield and pasta organoleptic characteristics.

It is important to point out that the data on the effects different fertilizers have on the mineral-substance concentrations in grain are quite contradictory. Some researchers have noted a lack of correlations between fertilizer application and mineral accumulation in grain due to complex interactions of several factors such as environmental conditions, genotype, fertilizer application rates, mechanized tillage, etc. (Jaskulska et al., 2018; Caldelas et al., 2023).

The main disadvantages of agronomic biofortification are the fertilizers have to be applied every season; and one has to take into account a number of additional factors such as soil structure, the amounts of essentials concentrated in it, lack or excess of precipitation, temperature conditions, biological uptake degree, and genotype influence (Kostin et al., 2020). According to I. Cakmak et al (2010), lack of adequate moisture level, high soil pH, high $CaCO_3$ content and low organic-matter concentration significantly reduce the availability and uptake of Zn and Fe from the soil, which prevents their optimum concentration in grain.

Another direction of agronomic biofortification is using soil microorganisms (*Bacillus*, *Azotobacter*, *Acinetobacter*, *Pseudomonas*, *Rhizobium*, etc.) for solubilization of mineral substances to enhance their mobility from soil to edible plant parts. It has been shown that seed inoculation or application of microorganisms directly into the soil lead to an increase in the concentration of such elements as Zn, Fe, Mn, Cu and Se

in wheat grain and shoots (Rana et al., 2012; Golubkina et al., 2017; Sun Z. et al., 2021; Ali M. et al., 2023). The mechanisms of microbial biofortification and the efficiency of the method for Fe and Zn uptake in various agricultural plants has been described in a review by S. Verma et al. (2021).

Apart from rhizosphere microorganisms, researchers have also experimented with arbuscular mycorrhizal fungi as an additional agent to improve the agronomically important traits of crops. The strains, either alone or in combination with rhizosphere microorganisms, increases the concentration of macronutrients (N and P), micronutrients (Zn and Fe) in wheat grain as well as wheat productivity parameters (1,000-grain weight; number of grains per ear; and number of productive tillers) (Ma et al., 2019; Yadav et al., 2020).

Despite the encouraging results obtained, a limited success has been achieved so far in this field due to the complexity of the interaction mechanisms between the microorganisms and the host plant and the influence of abiotic environmental factors such as soil mineral composition, temperature, and phytic acid effect on Zn and Fe bioavailability. The efficiency of microbial biofortification also significantly depends on the genotype, suggesting additional experiments are to be caried out to assess genotype responsiveness and select effective microbial strains (Garg et al., 2018).

Biofortification in Russia

Russia has seen practically no studies into wheat varieties to create the genotypes with increased content of mineral elements. To date, only limited data have been published on screening of domestic varieties and breeding lines for some micro- and macronutrients content and on the development of technologies for the use of fertilizers, growth regulators and microorganisms to improve the mineral composition of grain (Golubkina et al., 2017; Aristarkhov et al., 2018; Chikishev et al., 2020), e. g., Institute of Biology of the Karelian Scientific Center of RAS has been devising techniques for increasing Cu content in the root and shoots of *Triticum aestivum* L. and *Hordeum vulgare* L. (Kaznina et al., 2022).

As a part of Comprehensive Kazakh-Siberian Program under the Central Asia Sustainable Innovation Bureau (CASIB) umbrella, new varieties and breeding lines have been regularly screened for yield, grain, flour and baking qualities. As for the mineral composition, works in this area have just begun and the first data on the analysis of large collections of hexaploid wheat varieties and synthetic lines of different geographical origins have been published (Shamanin et al., 2021; Morgounov et al., 2022). The grains of the Russian varieties investigated under the CASIB program had higher Zn content than the varieties developed under the HarvestPlus program (Shamanin et al., 2021; Shepelev et al., 2022). However, the Russian-Kazakhstani samples were inferior to the genotypes from the USA and Japan in terms of Fe, Ca, Mo and Mg content.

Apart from breeding the varieties promising for functional nutrition, studies have been performed to produce purple-grained wheat. The presence of anthocyanins has shown not to affect the technological properties of bread, and adding purple-grain bran into flour has enriched bakery products with dietary fiber and anthocyanins (Fisenko et al., 2020). Nadira, a purple-grain variety of spring soft wheat is distinguished by increased antioxidant activity, disease resistance and high yield (Vasilova et al., 2021).

Studies have been initiated to identify genetic factors and map genes/QTLs in varieties of Russian origin as well as in synthetic, recombinant and introgression wheat lines (Morgounov et al., 2022; Potapova et al., 2023). First steps have been taken to develop genomic breeding models to improve the mineral composition of wheat grain (Potapova et al., 2024).

Conclusion

Biofortification is one of the modern and effective approaches aimed at enriching wheat grain with essential vitamins and minerals. Not only does it help to overcome the mineral elements deficiency in grain, but also to improve grain quality, yield and resistance to many diseases. The biofortification programs devised for the creation of new wheat genotypes with improved properties use different approaches, the main being traditional breeding that employs modern technologies of genetic mapping and agronomic techniques.

As for transgenesis and genomic editing, these technologies are still under development and have no current practical application. Genetic biofortification is considered to be more economically efficient and has a longer validity period than agronomic one. At present, the search for promising sources and donors for improving the mineral composition of wheat grain is supposed to be conducted in several directions: 1) study of variability of micro- and macronutrient concentrations among the ancient wheat varieties having greater genetic diversity if compared to modern ones; 2) search for new genetic loci in the germplasm of wheat relatives and creation of target gene donors with their participation; 3) development and use of the new DNA markers based on cereal genome sequencing data; 4) improvement of the gene/QTLs mapping methods employing bioinformatic approaches to identify the key candidate genes associated with mineral accumulation; 5) development of genomic breeding programs for targeted creation of biofortified genotypes. These methods of genetic fortification combined with optimal agro-technological methods will allow us to solve the problem of mineral nutrients deficiency in food.

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Exploitation of the genetic potential of *Thinopyrum* and *Agropyron* genera to protect wheat from diseases and environmental stresses

L.Ya. Plotnikova, V.V. Knaub

Omsk State Agrarian University named after P.A. Stolypin, Omsk, Russia
lya.plotnikova@omgau.org

Abstract. Common wheat is one of the most important food crops in the world. Grain harvests can be increased by reducing losses from diseases and environmental stresses. The tertiary gene pool, including *Thinopyrum* spp., is a valuable resource for increasing genetic diversity and wheat resistance to fungal diseases and abiotic stresses. Distant hybridization between wheat and *Thinopyrum* spp. began in the 1920s in Russia, and later continued in different countries. The main results were obtained using the species *Th. ponticum* and *Th. intermedium*. Additionally, introgression material was created based on *Th. elongatum*, *Th. bessarabicum*, *Th. junceiforme*, *Agropyron cristatum*. The results of introgression for resistance to diseases (leaf, stem, and stripe rusts; powdery mildew; *Fusarium* head blight; and *Septoria* blotch) and abiotic stresses (drought, extreme temperatures, and salinity) to wheat was reviewed. Approaches to improving the agronomic properties of introgression breeding material (the use of irradiation, *ph*-mutants and compensating Robertsonian translocations) were described. The experience of long-term use in the world of a number of genes from the tertiary gene pool in protecting wheat from leaf and stem rust was observed. *Th. ponticum* is a nonhost for *Puccinia triticina* (*Ptr*) and *P. graminis* f. sp. *tritici* (*Pgt*) and suppresses the development of rust fungi on the plant surface. Wheat samples with the tall wheatgrass genes *Lr19*, *Lr38*, *Sr24*, *Sr25* and *Sr26* showed defence mechanisms similar to nonhosts resistance. Their influence led to disruption of the development of surface infection structures and fungal death when trying to penetrate the stomata (prehaustorial resistance or stomatal immunity). Obviously, a change in the chemical properties of fungal surface structures of races virulent to *Lr19*, *Lr24*, *Sr24*, *Sr25*, and *Sr26* leads to a decrease in their adaptability to the environment. This possibly determined the durable resistance of cultivars to leaf and stem rusts in different regions. Alien genes with a similar effect are of interest for breeding cultivars with durable resistance to rust diseases and engineering crops with the help of molecular technologies.

Key words: wheat breeding; tertiary gene pool; *Thinopyrum*; *Agropyron*; introgression; resistance for disease and abiotic stresses; nonhost resistance; durable resistance.

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Использование генетического потенциала родов *Thinopyrum* и *Agropyron* для защиты пшеницы от болезней и абиотических стрессов

Л.Я. Плотникова, В.В. Кнауб

Омский государственный аграрный университет им. П.А. Столыпина, Омск, Россия
lya.plotnikova@omgau.org

Аннотация. Мягкая пшеница – одна из важнейших продовольственных культур в мире. Сборы ее зерна можно увеличить, сократив потери от болезней и стрессов окружающей среды. Третичный генофонд, включая виды рода *Thinopyrum*, является ценным ресурсом для увеличения генетического разнообразия и повышения устойчивости пшеницы к грибным заболеваниям и абиотическим стрессам. Отдаленная гибридизация между пшеницей и *Thinopyrum* spp. была начата в 1920-х гг. в России и позднее продолжена в разных странах. Основные результаты получены с использованием видов *Th. ponticum* и *Th. intermedium*. Дополнительно был создан интрогрессивный материал на основе видов *Th. elongatum*, *Th. bessarabicum*, *Th. junceiforme*, *Agropyron cristatum*.

В статье приведен обзор результатов применения генетического материала видов рода *Thinopyrum* для повышения устойчивости пшеницы к болезням (бурой, стеблевой и желтой ржавчине, мучнистой росе, фузариозу колоса и септориозным пятнистостям) и абиотическим стрессам (засухе, экстремальным температурам и засолению). Описаны подходы к улучшению агрономических свойств интрогрессивного селекционного материала (применение радиации, *ph*-мутантов и компенсирующих робертсоновских транслокаций). Проанализирован опыт длительной защиты пшеницы от листовой и стеблевой ржавчины в мире с помощью ряда генов третичного генофонда. Вид *Th. ponticum* является нехозяином для *Puccinia triticina* (*Ptr*) и *P. graminis* f. sp. *tritici* (*Pgt*) и подавляет развитие ржавчинных грибов на поверхности растений. Образцы пшеницы с пырейными генами *Lr19*, *Lr38*, *Sr24*, *Sr25* и *Sr26* проявляют защитные механизмы, сходные с механизмами нехозяев, что приводит к нарушению развития поверхностных инфекционных структур и гибели грибов при попытке внедрения в устьица (прегаусториальная устойчивость или устьичный иммунитет). Очевидно, изменение химических свойств поверхностных структур рас, вирулентных к *Lr19*, *Lr24*, *Sr24*, *Sr25* и *Sr26*, приводит к снижению их приспособленности к среде, что влияет на длительность устойчивости сортов к ржавчинным болезням. Чужеродные гены с аналогичным эффектом представляют интерес для селекции сортов с длительной устойчивостью к ржавчинным заболеваниям, а также конструирования культуры с помощью молекулярных технологий.
Ключевые слова: селекция пшеницы; третичный генофонд; *Thinopyrum*; *Agropyron*; интрогрессия; устойчивость к болезням и абиотическим стрессам; устойчивость нехозяев; длительная устойчивость.

Introduction

Cultivated wheat species, *Triticum aestivum* L. and *T. durum* Desf., are among the most important crops for world nutrition. It is assumed that the world's population will exceed 9.7 billion people by 2050. To provide nutrition for such a population, it is necessary to increase grain production to 900 million metric tons (Baker et al., 2020; Kumar et al., 2022). Common wheat has high plasticity, allowing it to be cultivated in most agricultural zones of the world. In this regard, wheat grain production has the most significant impact on global food security compared to other cereals (Kuzmanović et al., 2020; Kumar et al., 2022).

During centuries-old wheat breeding, most attention was paid to increasing yield and grain quality. As a result, a significant proportion of the genes determining adaptive capabilities to abiotic and biotic stresses was lost. Stressful environmental conditions and diseases lead to regular and significant losses of grain yield that can reach up to 20–40 % (Curtis, Halford, 2014). An increase in grain yield can be achieved by expanding the crop acreage, increasing the potential productivity by photosynthetic activity, and by reducing losses from abiotic and biotic factors (Savari et al., 2019; FAO Report, 2021). Increasing genetic diversity of wheat is actual for protection crop from diseases and stressful abiotic factors. *Thinopyrum* and related genera are promising sources for enrichment of wheat genetic pool and breeding of cultivars with improved properties.

Abbreviations
APR – adult plant resistance
ASR – all stage resistance
ROS – reactive oxygen species

Resistance gene symbols
Bdv – barley yellow dwarf virus
Fhb – *Fusarium* head blight
Lr – leaf rust
Pm – powdery mildew
Sr – stem rust
Snb – *Septoria nodorum* blotch
Stb – *Septoria tritici* blotch
Wsm – wheat streak mosaic virus
Yr – stripe rust

Impact of major diseases and abiotic stresses on wheat crops

During the 20th century, large scale wheat monocropping, often homogeneous in resistance to diseases, has been created on different continents. This situation has contributed to pathogen coevolution with plants in agroecosystems increasing over past 70 years (Zhan J.,McDonald, 2013). As a result, the appearance of new pathogens and virulent races within their populations has accelerated, and disease outbreaks have become more frequent (Chen X., 2005; Singh R.P. et al., 2016). The global burden of pathogens and pests on wheat production in 2010–2014 was estimated at 21.5 %, of which 18 % was determined by fungal diseases (Savari et al., 2019). The main losses in the amount of 15.1 % were determined by eight diseases (leaf, stem, and stripe rusts, *Septoria tritici* blotch and *Septoria nodorum* blotch, powdery mildew, *Fusarium* head blight, and tan spot) spread globally. Grain losses vary significantly between world regions, depending on climatic conditions, cultivar heterogeneity, and crop production technologies (McDonald, Stukenbrock, 2016; Singh R.P. et al., 2016).

Wheat is affected by leaf, stem, and stripe (yellow) rusts, caused by *Puccinia triticina* Eriks., *P. graminis* Pers. f. sp. *tritici* Eriks. et Henn, and *P. striiformis* Westend. f. sp. *tritici* Eriks., respectively. The common features of rust fungi are high reproduction, variability, and airborne dispersal of urediniospores, often over long distances, to new regions and even continents (McDonald, Stukenbrock, 2016; Savari et al., 2019). *P. triticina* is the most plastic species among wheat rust fungi and regularly affects common wheat crops in many world regions (Kolmer, 2013). In the last decade, leaf rust has increased significantly in the main wheat production regions in China and India (Gao et al., 2019; Aravindh et al., 2020). Wheat stem rust development was suppressed worldwide

in the second half of the twentieth century due to the use of cultivars carrying the *Sr31* gene transferred from cereal rye, *Secale cereale* L. (Singh R.P. et al., 2015). However, in Uganda in 1998, the Ug99 race (TTKSK) appeared, which overcame the *Sr31* gene, and later other races unrelated to Ug99 (such as Digalu, and Sicilian) appeared. Over two decades, stem rust accelerated in Africa, the Middle East, and in Western Europe (Singh R.P. et al., 2015; Patpour et al., 2022).

Wheat stripe rust used to spread in regions with a cool and humid climate. However, following the appearance of *P. striiformis* f. sp. *tritici* clones adapted to high temperatures, there was a rapid spread of the pathogen to new regions. Since the 2000s, stripe rust has become a new threat to grain production in many regions, and regular outbreaks now occur in North and South America, Africa, Northwest Europe, India, China and Russia (Ali S. et al., 2017; Gultyaeva et al., 2022). FAO claims that rusts are the most destructive transborder wheat diseases, making them serious threats to food security worldwide (Singh et al., 2016; FAO Report, 2021).

Another important global wheat disease is powdery mildew, caused by *Blumeria graminis* f. sp. *tritici* (DC.) Speer. Previously, powdery mildew, while affecting wheat crops worldwide, prevailed in regions with damp and cool climate. During recent decades, the disease has increased in warmer regions, especially when using intensive technologies with high doses of nitrogenous fertilizers (Zhang R.Q. et al., 2020; Yang G. et al., 2023). Largest crop losses were noted in China, Northwest Europe, and India (Savari et al., 2019).

Septoria blotch diseases are caused by a complex of fungi, the main of them are *Zymoseptoria tritici* (Roberge ex Desm.) Quaedvl. & Crous. (= *Septoria tritici* Desm.), and *Parastagonospora nodorum* (Berk.) Quaedvl. (= *Septoria nodorum* (Berk.). Significant negative effect of the *Septoria* fungal complex on wheat crops has been noted since 1980. In previous decades, *Septoria tritici* blotch caused high grain losses in humid coastal regions of Europe and North America (O'Driscoll et al., 2014; Fones, Gurr, 2015). During the last decade, *Septoria tritici* blotch has spread to the arid regions of Africa, Northern Kazakhstan, and Western Siberia (Babkenova et al., 2020; Tadesse et al., 2020; Plotnikova et al., 2023b). On the territory of Russia, wheat leaf and ear *Septoria* diseases are mainly caused by two species – *Z. tritici* and *P. nodorum*, and the ratio of pathogens varies significantly depending on the region (Toropova et al., 2020).

Fusarium head blight (FHB) is caused by *Fusarium graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schwein.)]. FHB impacts include wheat yield loss, deterioration of grain quality, and mycotoxin contamination, effecting human and animal health (Alisaac, Mahlein, 2023). Frequent FHB epidemics have been occurring since the 1990s in the USA, Canada, South America, China (Zhu et al., 2019; Alisaac, Mahlein, 2023). Tan spot (yellow spot, yellow leaf spot) is caused by the necrotrophic fungus *Pyrenophora tritici-repentis* (Died.) Dreches [anamorph *Drechslera tritici-repentis* (Died.) Shoemaker]. The first tan spot epidemics were reported in the 1970s in North America, Australia, and Southern Africa, and later the disease spread globally (Carmona et al., 2006; Phuke et al., 2020).

Climate change is a threat to sustainable crop production. According to the FAO report, the number of disasters (climatological, hydrological, biological and geophysical) per year by decade grew from 90 in 1970s to 360 in 2010s (FAO Report, 2021). The largest increase was noted for weather-related disasters, such as drought, storms, and extreme temperatures. Agriculture is especially vulnerable to increased frequency and intensity of extreme weather-related and climate induced disasters. Damage and loss in agriculture for 2008–2018 was estimated at 63 % (FAO Report, 2021). Among abiotic stresses, drought, extreme temperatures, and soil salinity have the greatest negative impact on wheat grain production (Kosová et al., 2014; Ali N., Mujeeb-Kazi, 2021). About one third of areas most suitable for agriculture, located in the warm regions, are becoming more arid (Goncharov, 2021). Losses in grain production in the world related to a lack of precipitation and extreme temperatures worldwide can reach 28 % (Kumar et al., 2022). Winter varieties produce higher yields compared to spring genotypes. However, to expand winter crops to risky farming areas, it is necessary to improve their winter hardiness (Fisenko, Kuzmina, 2020). The wheat growing area can also be increased by using saline land. However, this requires the breeding of wheat cultivars with high salt tolerance (Yang Z. et al., 2022). The use of a wide range of new genes in breeding is the basis for sustainable defence of bread and durum wheat from stresses (Ceoloni et al., 2014).

Enhancement of genetic diversity of wheat with alien gene pools

Genetic protection of cultivars is considered the most cost-effective and environmentally friendly way to control diseases (Singh R.P. et al., 2016; Gultyaeva et al., 2022). Wild and cultivated cereal species are the main reservoirs of valuable genes for wheat breeding (Ceoloni et al., 2014; Kumar et al., 2022). B. Friebe and co-workers (1996) proposed to divide plant species into primary, secondary, and tertiary gene pools by availability for breeding. Species in primary gene pool have genomes that are homologous to the subgenomes of common wheat (AABBDD), and secondary gene pool has at least one subgenome homologous to wheat. The genetic material of the primary and secondary gene pools can be transferred to common wheat relatively easily in the form of short translocations by recombination of homologous chromosomes.

Tertiary gene pool species have genomes that differ from common wheat subgenomes (homoeologous). Introgression of genetic material from tertiary gene pool species to wheat is difficult due to the differences in homoeologous chromosome structure, thus requiring special methods (Friebe et al., 1996; Li H., Wang 2009). The tertiary gene pool includes the genera *Aegilops* L. (species with U, C, M, T, X genomes), *Secale* L., *Hordeum* L., *Thinopyrum* Á. Löve, *Agropyron* Gaertn., *Leymus* Hochst., *Haynaldia* L. (= *Dasypyrum*), and *Pseudoroegneria* (Nevski) A. Löve (Friebe et al., 1996; Kroupin et al., 2019; Kumar et al., 2022). During the 20th century, the most attention in the world was paid to *Thinopyrum* spp. Perennial *Thinopyrum* spp. are the components of natural ecosystems and pastures in different Eurasian regions (Tsvelev, 1984; Li H., Wang, 2009). In this regard, they are well adapted to

contrasting climatic conditions and have a wide spectrum of resistance genes to abiotic and biotic stresses (Lammer et al., 2005; Li X. et al., 2017).

Scientific research of the genetic potential of *Thinopyrum* and related genera and the implementation of the results in practice have been ongoing for more than a century (Table 1). The first successful work on distant hybridization between wheat and *Thinopyrum* spp. (before *Agropyron*) was carried out by N.V. Tsitsin in Russia during 1920–1930 (Tsitsin, 1979). A set of species was tested, of which two species were most promising for further work, viz. intermediate wheatgrass *Thinopyrum intermedium* Barkworth & D.R. Dewey and the tall wheatgrass *Th. ponticum* (Podp.) Z.-W. Liu & R.-C. Wang. It should be noted that the classification of these species has changed many times and is still in the process of formation. In this regard, different names of these species are found in publications of different periods, e. g. *Th. intermedium* (= *Agropyron intermedium* (Host) Beauv, *Ag. glaucum*), and *Th. ponticum* (= *Th. ponticum* (Podp.) Barkworth et D.R. Dewey, *Ag. elongatum* (Host) Beauv, *Lophopyrum ponticum* (Popd.) A. Löve, *Elytrigia pontica* (Popd.) Holub.). At the beginning, both wheatgrass species were selected based on perennial habit and tolerance to abiotic stresses (frost resistance, winter hardiness, and drought tolerance). Later, *Thinopyrum* spp. were recognized as valuable reservoirs of genes for resistance to diseases, and most studies were devoted to this problem (Friebe et al., 1996; Li H., Wang, 2009). At the first stage, the intergeneric amphiploids were obtained, which were crossed with *T. aestivum*, and later partial amphiploides (named Wheat-Wheatgrass Hybrids, WWHs) with different sets of wheatgrass chromosomes were produced. Some stable WWHs were selected and used as commercial forage regrowing and perennial cultivars (Tsitsin, 1979).

Later, on their basis, winter-hardy wheat cultivars were bred for the European part of Russia (Upelniek et al., 2012). Currently, Tsitsin's heritage is maintained in the form of octaploid partial amphiploid WWHs collection ($2n = 56$, including 42 chromosomes of common wheat, and 14 chromosomes from different *Thinopyrum*'s subgenomes) (Main Botanical Garden of the Russian Academy of Sciences, Moscow, Russia) (Upelniek et al., 2012; Kroupin et al., 2019).

Work on the introgression of the *Thinopyrum* spp. genetic material into the wheat gene pool was continued in Russia in 1980–2010s. Breeding material on the base of the Avrocum amphiploid was created at the P.P. Lukyanenko National Grain Center (Krasnodar, Russia). Avrocum was obtained by the hybridization of the tetraploid form tetra-Avrora (common wheat cv. Avrora without the D genome) with *Th. intermedium* (Davoyan et al., 2016). At the same time, amphiploids based on *Th. intermedium* and later wheat lines with 6D chromosomes substituted by one of the homoeologous chromosomes 6Agi (6Agi or 6Agi2) were obtained. Later, introgression spring common wheat cultivars with alien 6Agi chromosomes were bred at the N.M. Tulaikov Samara Research Institute of Agriculture and at the Federal Centre of Agriculture Research of the South-East Region (Samara and Saratov, accordingly; Russia) for the Volga region (Salina et al., 2015; Sibikeev et al., 2017). New introgression lines of spring common wheat resistant to rusts and *Septoria* blotch diseases were bred on the basis of *Th. ponticum* at Omsk State Agrarian University (Omsk, Russia) (Plotnikova et al., 2014, 2021, 2023b).

Introgression of the genetic material of *Thinopyrum* spp. was carried out independently in different countries of the world (Table 1). In 1930–1940, distant hybridization of *T. aestivum* with *Th. intermedium* and *Th. ponticum* was realized by W.J. Sando in the USA, and a collection of partial amphiploids was obtained (Sando collection, USDA, National Small Grains Germplasm Research Facility, Aberdeen, Idaho, USA) (Hang et al., 2005). Similar work was carried out in Germany and Canada (Peto, 1936; Smith D.C., 1943). Great attention was paid to the introgression of *Thinopyrum* spp. genetic material in China after the 1950s. S.C. Sun created partial amphiploids with *Th. intermedium* ("Zhong" series), and later, common wheat cultivars were created on their basis (Sun et al., 1981). Z.S. Li produced a set of partial amphiploids on the base of *Th. ponticum* with resistance to leaf and stripe rusts (e. g. Xiaoyan series, including Xiaoyan 68, Xiaoyan 693, Xiaoyan 7430, Xiaoyan 7631, and Xiaoyan 784) (Li Z.S. et al., 2008). Over time, other series of partial amphiploids were created in different countries: Agrotana (Chen Q. et al., 1995), PMV (Fedak et al., 2000), SS (Oliver et al., 2006), BE-1 (Sepsi et al., 2008), SN (He F. et al., 2017). The partial amphiploids were used for the breeding of numerous supplemented, substituted, and introgression lines with translocations of various size.

The international classification of *Thinopyrum* spp. is in progress and is being refined using methods of molecular cytogenetics. According to current concepts, the *Thinopyrum* genus includes species with a wide range of genomes and ploidies (from diploids to decaploids). The diploid accessions ($2n = 2x = 14$) were identified in *Th. elongatum* D.R. Dewey and *Th. bessarabicum* (Savul & Rayss) Á. Löve. The tetraploids ($2n = 4x = 28$) were determined in *Th. junceiforme* Á. Löve, and the hexaploids were among *Th. intermedium* and *Th. junceum* ($2n = 6x = 42$). The decaploids were found among *Th. ponticum* accessions (Chen S. et al., 2013; Mo et al., 2017). Based on cytogenetic and molecular genetic studies, the genomic composition of the hexaploid *Th. intermedium* is JJJ^sJ^sStSt (Chen Q. et al., 1998) or $EEE^{st}E^{st}StSt$ (Wang R.R.-C., 2011), and the decaploid *Th. ponticum* formula is $JJJJJ^sJ^sJ^sJ^sJ^s$ (Chen Q. et al., 1998) or $EEE^bE^bE^xE^xStStStSt$ (Zhang X. et al., 1996). The classification of genomes proposed by R.R.-C. Wang (2011) is most often used, but in some articles genome designation J was used. The J or J^s subgenomes are highly homologous with the genomes E^e of *Th. elongatum* and E^b of *Th. bessarabicum*, accordingly. The St subgenome is closely related to the genome of *Pseudoroegneria strigosa* (Chen Q. et al., 1998; Wang L. et al., 2017). *Agropyron* spp. have a different number of P genomes ($2n$–$6n$) (Wang R.R.-C., 2011).

Developing methods for effective introgression of alien genetic material and improving the properties of breeding material

When transferring the genetic material from tertiary gene pool species to wheat, difficulties arise at different stages of the work. Common problems include difficulties in intergeneric crossing, F_1 hybrid sterility, and lack of homeologous

Table 1. The main stages of introgression of the genetic material from *Thinopyrum* and related genera to the wheat gene pool

Period	Country	Scientific result	Practical result	References
1920–70s	Russia	Study of *Thinopyrum* spp. Identification of promising species Amphiploids on the base of *Th. ponticum* and *Th. intermedium*, and partial amphiploids WWHs Collection of WWHs (2*n* = 56)	Cultivars of forage regrowing and perennial WWHs. Hardy winter common wheat cultivars	Tsitsin, 1979; Upelniek et al., 2012; Kroupin et al., 2019
1930–40s	USA	Amphiploids on the base of *Th. ponticum* and *Th. intermedium* Partial amphiploids "Sando collection"	–	Hang et al., 2005
	Germany	Amphiploids on the base of *Th. ponticum* and *Th. intermedium* Collection of partial amphiploids	–	Peto, 1936
	Canada	Amphiploids on the base of *Th. ponticum* and *Th. intermedium* Collection of partial amphiploids	–	Smith D.C., 1943
1950–70s	China	Partial amphiploids based on *Th. intermedium*, "Zhong" series Partial amphiploids based on *Th. ponticum*, "Xiaoyan" series	Common wheat cultivars	Sun, 1981; Li Z.S. et al., 2008
1950–80s	USA	Developing methods for effective introgression using γ-irradiation, ph-mutants, Robertsonian translocations Introgression lines Transfer of *Lr19*/*Sr25*, *Sr24*/*Lr24*, *Sr26*	Common wheat cultivars	Sears, 1956, 1976; Knott, 1968; Smith E.L. et al., 1968; Friebe et al., 1994, 1996, 2000
1980–2010s	USA China Germany, France	Partial amphiploids based on *Th. ponticum* and *Th. intermedium* Collections of partial amphiploids "Agrotana", PMV, SS, BE-1, SN	Breeding material	Chen Q. et al., 1995; Fedak et al., 2000; Oliver et al., 2006; Sepsi et al., 2008; He F. et al., 2017
1990–2010s	USA China	Transfer of *Lr38*, *Sr43*, *Sr44*, *Pm40*, *Pm43*, *Bdv2*, *Bdv3*	Common wheat cultivars	McIntosh et al., 1995, 2018
1980–2010s	Russia	Partial amphiploids based on *Th. intermedium*, lines with substituted 6D-6Agi chromosomes Partial amphiploids based on *Th. ponticum* Introgression lines	Common wheat cultivars Breeding material	Davoyan et al., 2016; Salina et al., 2015; Sibikeev et al., 2017 Plotnikova et al., 2023b, c
1990–2010s	USA China	Study of genome constitution of *Thinopyrum* spp. and relative genera Lines with supplemented and substituted chromosomes, different translocations	Breeding material Common wheat cultivars	McIntosh et al., 1995, 2018; Zhang X. et al., 1996; Chen Q. et al., 1998; Chen S. et al., 2013; Mo et al., 2017; Guo X. et al., 2023
1990–2010s	USA China	Introgression genetic material from relative species *Th. bessarabicum*, *Th. elongatum*, *Th. junceiforme*, *Th. distichum*, *A. cristatum*	Breeding material	Qi et al., 2010; Chen S. et al., 2013; Zhang Z. et al., 2017; Jiang et al., 2018; Li W. et al., 2019
1980–2010s	USA Russia	Domestication of *Th. intermedium*	Fodder intermedium wheatgrass cultivars	Pugliese et al., 2019 Bajgain et al., 2020 Pototskaya et al., 2022
2010–2020s	USA China Germany	Genome sequencing Cloning of *Sr26*, *Sr61*, *Fbh7* genes	Breeding material	Arora et al., 2019; Wang H. et al., 2020; Frailie, Innes, 2021; Zhang J. et al., 2021

Note. WWHs – wheat-wheatgrass hybrids.

chromosomes conjugation. As a result of poor conjugation between homoeologous chromosomes, large alien fragments (e. g. whole chromosomes, chromosome arms, or large terminal translocations) are usually transferred into introgression lines (Liu J. et al., 2013; Leonova, 2018). Consequently, the material obtained by distant hybridization is significantly inferior in properties to commercial varieties (Friebe et al., 1996; Li H., Wang et al., 2009). The major reasons for the deterioration of wheat properties is a close linkage between target and undesirable genes (linkage drag), or insufficient genetic complementation between the alien fragment and the wheat genome, and the fact that alien translocation does not compensate the absence of wheat genetic material (Wulff, Moscou, 2014; Hao et al., 2020). In many cases, wheat traits decrease is due to the fact that a large alien fragment does not compensate the loss of important genetic material (Friebe et al., 2005).

In the 1950–1970s, methods were developed that increased the introgression efficiency from relative species to wheat. For the first time, the procedure was implemented when transferring the genetic material from *Ae. umbellulata* (UU) to common wheat (Sears, 1956). At the first step, an interspecific hybrid was obtained, and its chromosomes were doubled using colchicine. The resulting amphidiploid was backcrossed with wheat, and a substituted line was obtained, which was used as a genetic bridge to transfer leaf rust resistance to common wheat. To facilitate the transfer of genetic material between the U and wheat chromosomes, the pollen of the substituted line was irradiated to induce multiple chromosome breaks followed by recombination of the fragments. As a result of the work, the cv. Transfer was bred, carrying the *Lr9* gene for resistance to leaf rust (Sears, 1956).

Later, after the research on wheat meiosis genetic control, it was found that wheat chromosome 5B carries the *Ph* gene that suppresses the homoeologous chromosomes conjugation. To induce conjugation, it is possible to cross introgression lines with aneuploids in 5B chromosome or to use mutants with the *ph* genes. The mutant *ph1b* gene (or similar *ph2a* and *ph2b*) facilitates loci exchange between homoeologous chromosomes (Sears, 1976). Similar effects may be supplied by the chromosome 5P of *A. cristatum* (PPPP) and some accessions of *Ae. speltoides* (SS) (Friebe et al., 2000; Han et al., 2023).

The hexaploid *Th. intermedium* crosses relatively easily with common wheat (the average seed setting is about 24 %), and amphiploids can be obtained by direct crossing (Mo et al., 2017). To transfer the genetic material from the decaploid *Th. ponticum*, amphiploids with the tetraploid wheat *T. turgidum* (AABB) need to be created (Tsitsin, 1979). Partial amphiploids with chromosome combinations from various *Thinopyrum* spp. subgenomes were obtained after backcrossing to common wheat (Friebe et al., 2000; Li H., Wang, 2009). In rare cases, the transfer of homeologous fragment occurs by spontaneous translocation (Knott, 1968). But the induction of translocations using irradiation or induced homeologous recombination with *ph1b* mutants is more effective for transferring small loci to wheat genome (Sears, 1978). Using these methods, the transfers of at least 134 loci from the *Th. ponticum* to common wheat were maid (Baker et al., 2020). Recently, a large set of lines with wheatgrass introgressions of various size were bred (Mo et al., 2017; Kroupin et al., 2019).

The transfer of multiple alien fragments and location of introgressions at various chromosomes and their arms is possible (Table 2). For example, *Lr38* gene in *Th. intermedium* derivatives was transferred to four wheat chromosomes (2A, 5A, 3D and 6D) (Friebe et al., 1996). The loci with *Sr24*/*Lr24* genes from the *Th. ponticum* were identified in two wheat chromosomes (1B and 3D). The loci with *Lr19*/*Sr25* genes were identified in different arms of the 7D chromosome (McIntosh et al., 1995; Friebe et al., 1996). Introgression lines with the best properties were selected for breeding the cultivars.

The improvement of the properties of some introgression lines was achieved by reducing alien fragment size using γ-irradiation of seeds, plants, and pollen, or induced homeologous recombination with *ph1b* mutants (Sears, 1978). The limited application of *Lr19*/*Sr25* from *Th. ponticum* in breeding was associated with its linkage with the *Y* gene determined yellow flour color. *Lr19/Sr25* and *Y* genes were separated using *Ph1* deletion lines (Zhang W. et al., 2005). The valuable *Fhb7* gene conferring resistance to both *Fusarium* head blight and *Fusarium* crown rot was tightly linked with the *PSY-E2* gene that determines yellow flour color. Using the *ph1b* mutant, lines with shortened translocations devoid of the *PSY-E2* gene were obtained (Li M.Z. et al., 2022). Thanks to *ph1*-induced homoeological recombination, interspecific gene transfer from *T. aestivum* to *T. durum* was achieved. Three loci with alien *Lr19/Sr25/Y* and *Pm13* genes (from *Th. ponticum* and *Ae. longissima*, respectively), and *Gli-D1/Glu-D3* (effecting gluten properties) were transferred from common wheat chromosomes 7D, 1D and 3B to durum wheat arms 7AL, 3BS and 1AS (Kuzmanović et al., 2020).

In some cases, noncompensating wheat-alien translocations occur in introgressive material. This is due to the participation in meiosis of homoeologous chromosome arms, which differ in gene sets and their order. Noncompensating translocations provoke genomic duplications or deficiencies, which lead to line genetic instability and prevent the use of a valuable gene in breeding (Friebe et al., 1996). Compensating Robertsonian translocations (RobTs) are used for functional substitution of lost fragments to correct wheat genotypes (Friebe et al., 2005). Such a method was used to improve the properties of the line with the *Sr44* gene from *Th. intermedium*. As a result, the compensating RobT in the form of recombination T7DL×7J#1S was identified, consisting of wheat arm 7DL translocated to the *Th. intermediate* arm 7J#1S (Liu W. et al., 2013). Similar works were carried out, developing lines with the *Sr51*, *Sr52*, and *Sr53* genes (from *Ae. searsii* Feldman & Kislev ex Hammer, and *Ae. geniculata* Roth, respectively) (Liu W. et al., 2011a, b).

In the rarest cases, substitution of wheat chromosomes by alien ones does not decrease agronomic properties. This was the case for spring common wheat cultivars with chromosome 6D substituted by 6Ai or 6Ai2 from the *Th. intermedium* J(=E) subgenome. A set of cultivars with the 6Ai or 6Ai2 chromosomes (homologous) were bred in the Russian Volga

Table 2. Results of introgression of genetic material from *Thinopyrum* spp. to wheat gene pool

Donor species	Gene, trait	Translocation, substituted chromosome	Cultivar, line	References
Th. ponticum	*Lr19*/*Sr25*	7DL-7Ae#1L	Agatha	Knott, 1968
		7D-7Ag no.1	Sears's transfer	Sears, 1978
	Sr24/*Lr24*	3DL-3Ae#1L	Agent	Smith E.L. et al., 1968
		3D-3Ag#1	Sears's transfer	Sears, 1978
		T1BL·1BS-3Ae#1L	Amigo	McIntosh et al., 1995
	Sr26	T6AS.6AL-6Ae#1L	*Sr26*/9*LMPG	Friebe et al., 1994; McIntosh et al., 1995
		6A-6Ae#1L	Thatcher	McIntosh et al., 1995
	Sr unknown	T5DS·5DL	WTT34	Yang G. et al., 2021
	Yr69	2AS	CH7086	Hou et al., 2016
	YrTp1, *YrTp2*	2BS, 7BS	A-3	Yin et al., 2006
	Yr unknown, APR	5A-St	ES-7	Mo et al., 2017
	Yr unknown	1B-1J^{S}	SN19647	Wang Y.Z. et al., 2020
	Pm51	2BL	CH7086	Zhan H.X. et al., 2014; Hou et al., 2016
	Pm unknown	T5DL·5AgS	11-20-1	Li X. et al., 2017
	Pm unknown	T1BL·1RS	SN0293-7	Li M.Z. et al., 2022
	Pm unknown	1B-1J^{S}	SN19647	Li M.Z. et al., 2021
	Pm unknown	1D-1J^{S}	CH10A5	Wang Y.Z. et al., 2020
	Pm unknown, APR	4D-4AgS	Blue 58	Yang G. et al., 2023
	Fhb7	7DL-7Ae#1L	SDAU1881	Ceoloni et al., 2017; Wang H. et al., 2020
	Stb unknown	?	2/2015, 337/2017	Plotnikova et al., 2023b
	Snb unknown	?	6/2015, 322/2017	
Th. intermedium	*Lr38*	T1DS·1DL-7Ai#2L	T25	McIntosh et al., 1995
		T2AS·2AL-7Ai#2L	W49 (=T33)	
		T3DL·3DS-7Ai#2L	T4	
		T5AL·5AS-7Ai#2L	T24	
		T6DS·6DL-7Ai#2L	RL6097	McIntosh et al., 2018
	Lr6Agi	6D-6Agi	Multi 6R	Salina et al., 2015; Sibikeev et al., 2017
		6D-6Agi2	Tulaikovskaya 5	
	Sr43	7D,7DS-7el$_2$S.7el$_2$L	KS10-2, KS23, KS24, KS24-2	Niu et al., 2014
	Sr44	T7DS-7Ai#1L·7Ai#1S	Lines 86.187, TA5657	McIntosh et al., 2018
	Yr50	4BL	CH233	Liu J. et al., 2013; McIntosh et al., 2018
	YrYu25	?	Yu25	Wang S. et al., 2022
	Yr unknown	J or J^{S}	Z4	Huang et al., 2014
	YrT14	7J or 7J^{S}	Zhongke 15, Zhongke 78	Guo X. et al., 2023
	YrL693	?	L693	Huang et al., 2014
	*Yr*CH-1BS	T1BL.1BS-3Ai	CH-1BS	Zheng X. et al., 2020
	Pm40	7BS	GRY19	Luo et al., 2009b

Table 2 (end)

Donor species	Gene, trait	Translocation, substituted chromosome	Cultivar, line	References
	Pm43	2DL	CH5025	He R.L. et al., 2009
Th. intermedium	*Pm/Yr* unknown	T6BS.6AiL	CH13-21	Zhan H. et al., 2015
	Bdv2	T7DS·7DL-7Ai#1L 7DS-7Ai#1S·7Ai#1L T1BS-7Ai#1S·7Ai#1L	TC14, TC5, TC6, TC8, TC9, TC7	Hohmann et al., 1996; McIntosh et al., 2018
	Bdv3	7DS·7DL-7EL	P961341	Ohm et al., 2005
	Wsm1	T4DL·4Ai#2S	CI 17884	Wells et al., 1982
		T4AL·4Ai#2S	KS93WGRC27	Gill et al., 1995
		A29-1-13-2	CI 17766	McIntosh et al., 2018
Th. bessarabicum	Salt tolerance	T2JS-2BS·2BL	TJ04	Qi et al., 2010
Th. elongatum	*Fhb-7EL* *Pm*	? ?	DS7E ?	Chen S. et al., 2013
Th. junceiforme	Salt tolerance *Fhb* unknown *Wsm* unknown	?	Introgression lines	Li W. et al., 2019; Singh D. et al., 2019

region (Multi 6R, Belyanka, Voevoda, Lebedushka, Tulaikovskaya 5, Tulaikovskaya 100, Tulaikovskaya Zolotistaya, and others). These cultivars showed broad spectrum resistance to leaf and stem rusts, high yield and grain quality, and drought tolerance (Salina et al., 2015; Sibikeev et al., 2017). In China, the big achievement was the breeding of the cv. Xiaoyan 6 with double translocations from *Th. ponticum* on the chromosomes 2A and 7D. The cv. Xiaoyan 6 was multi-resistant to fungal diseases, had high yield, grain quality, and environmental plasticity. The cv. Xiaoyan 6 was widely cultivated in the 1980–1990s, and used as a parent for more than 60 common wheat cultivars (Zhang X. et al., 2011).

Contribution of *Th. ponticum* and *Th. intermedium* as sources of useful genes

When introgression of the genetic material of *Thinopyrum* spp., the main attention was paid to emerging disease challenges, and the studies have become more intensive in recent decades. During 1960–2020, a set of designated resistance genes to leaf, stem, and stripe rusts was transferred from *Th. ponticum* to *T. aestivum*. Some of these genes are closely linked and are present in complex translocations, viz. *Lr19*/*Sr25*, *Sr24*/*Lr24*, and others are single, viz. *Lr29*, *Sr26*, *Sr43*, *Sr61* (=*SrB*), *Yr69* (Table 2) (McIntosh et al., 1995, 2018).

After stem and stripe rusts progressed in 2000s, the cereal species and amphiploid collections were screened for disease resistance. Screening of the five *Thinopyrum* spp. (242 accessions) showed, that *Th. ponticum* and *Th. intermedium* are highly resistant to Ug99 race (Zheng Q. et al., 2014a, b). Partial amphiploids created in China in the 1950s (Xiaoyan 68, Xiaoyan 7430, and Xiaoyan 784) are highly resistant to Ug99 group races (Zheng Q. et al., 2014b). A new introgression line, WTT34, was created, carrying at least one new *Sr* gene in the T5DS·5DL translocation (Yang G. et al., 2021). Based on the Xiaoyan 784 amphiploid, the ES-7 line was created with 5A-St substituted chromosomes carrying adult plant resistance (APR) to stripe rust (Mo et al., 2017). *Yr69* gene was transferred from the Xiaoyan 7430 amphiploid to wheat chromosome arm 2AS (Hou et al., 2016). In the A-3 line, two putatively new stripe rust resistance genes, *YrTp1* and *YrTp2*, were identified in the chromosome arms 2BS and 7BS, respectively (Yin et al., 2006). Additional undesignated genes were determined in other lines (Zheng Q. et al., 2014a; Wang Y.Z. et al., 2020).

The potential of the genus *Thinopyrum* is poorly used to protect wheat from powdery mildew. Currently, only *Pm51* (among 65 designated genes) has been transferred from *Th. ponticum* (McIntosh et al., 2018). *Pm51* confers broad-spectrum all-stage resistance (ASR) to the disease (Zhan H.X. et al., 2014). New unknown *Pm* genes were identified in lines SN19647 and CH10A5, in which 1B and 1D chromosomes were replaced by $1J^s$ chromosomes (Wang Y.Z. et al., 2020; Li M.Z. et al., 2021). Lines 11-20-1 (with the T5DL·5AgS translocation) and SN0293-2 showed ASR resistance to a set of races (Li X. et al., 2017; Li M.Z. et al., 2022). In the blue-grained wheat line Blue 58 with a chromosome 4Ag(4D) substitution, a gene(s) for APR was present in the short arm of 4Ag that has determined resistance to powdery mildew for over forty years (Yang G. et al., 2023).

In addition to diseases caused by biotrophic fungi (rusts and powdery mildew), an increase of diseases caused by hemibiotrophic, necrotrophic and viral pathogens has been noted. *Septoria* blotch diseases cause significant losses in grain yield. Crops are mainly protected by the use of fungicides, and genetic protection is poorly implemented (Fones, Gurr, 2015). Currently, there are no resistance genes to *Septoria*

blotches transferred from *Thinopyrum* spp. among the identified ones. Resistance to *Septoria nodorum* blotch, *Fusarium* head blight and tan spot was revealed in the interspecific hybrid *Th. ponticum* × *Th. intermedium* (Oliver et al., 2006). Among introgression lines with genetic material of *Th. ponticum* bred in Western Siberia, a set of lines highly resistant to *Septoria tritici* blotch and *Septoria nodorum* blotch, with unknown genes (*Stb* and *Snb*, accordingly), were determined (Plotnikova et al., 2023b). Additionally, from *Th. ponticum*, some resistance genes were transferred, viz. to *Fusarium* head blight (*Fhb7*), common root rot, barley yellow dwarf virus (*Bdv*), wheat streak mosaic virus (*Wsm*) (Ceoloni et al., 2017; Kumar et al., 2022).

Tall wheatgrass has also been used as a source of valuable traits for wheat, such as resistance to pre-harvest sprouting (Kocheshkova et al., 2017), blue aleurone layer (Liu L.Q. et al., 2018), frost resistance, winter hardiness (Upelniek et al., 2012), and drought tolerance (Kuzmanović et al., 2016; Plotnikova et al., 2023c).

Thinopyrum intermedium is the source of the rust and powdery mildew resistance genes *Lr38*, *Sr43*, *Sr44*, *Yr50*, *Pm40* and *Pm43* (McIntosh et al., 1995, 2018; Friebe et al., 1996; He R.L. et al., 2009; Luo et al., 2009a, b; Liu J. et al., 2013; Niu et al., 2014). New genes *Lr6Agi* and *Sr6Agi* were identified in the substitution chromosomes 6Agi and 6Agi2 (Salina et al., 2015; Sibikeev et al., 2017). Lines with stripe rust resistance gene *YrYu25* were obtained based on amphiploid TAI7047 (Luo et al., 2009a). Four genes for resistance to stripe rust were identified in the *Th. intermedium* St subgenome (chromosomes 1St, 2St, 3St, and 7St) (Wang S. et al., 2022), and one gene was determined in subgenome J or J^s (in the short arm of the supplemented chromosome of line Z4) (Lang et al., 2018). Lines Zhongke 78 and Zhongke 15 with the *YrT14* gene in translocations from the alien 7J or $7J^s$ chromosome were bred in China (Guo X. et al., 2023). The *YrL693* gene was reported in introgression line L693 (Huang et al., 2014). Potentially new genes for resistance to stripe rust (*YrCH-1BS*) and powdery mildew were determined in lines with T1BL.1BS-3Ai and T6BS.6AiL translocations (Zhan H. et al., 2015; Zheng X. et al., 2020). *Th. intermedium* was also a good source of resistance genes to barley yellow dwarf virus (*Bdv2*, *Bdv3*), and wheat streak mosaic virus (*Wsm1*) (Wells et al., 1982; Gill et al., 1995; Hohmann et al., 1996; Ohm et al., 2005; Li H., Wang 2009; McIntosh et al., 2018).

Thinopyrum intermedium can be used not only as a reservoir of genes for improving the food common wheat, but also as a pasture and forage crop. In the 1980s, the work began on domestication of intermedium wheatgrass (Bajgain et al., 2020; Pototskaya et al., 2022). For forage crops, some features are valuable, e. g., perennial habit, rapid regrowth after cutting or grazing, resistance to frost and diseases, and improved feed quality (Hassani et al., 2000; Lammer et al., 2005). As a result of long-term work, perennial intermediate wheatgrass cultivars (Kernza, MN-Clearwater, Sova) were bred for fodder grain and dual-use (for grain and hay) (Hassani et al., 2000; Bajgain et al., 2020; Pototskaya et al., 2022). These cultivars are of interest as gene reservoir for the breeding of wheat cultivars for various purposes.

Introgression of genetic material of diploid and tetraploid *Thinopyrum* spp. and relative species to wheat gene pool

Despite great achievements in distant hybridization, introgression from polyploid heterogenomic species is a complex problem. In this regard, diploid and tetraploid species with genomes similar to the *Th. intermedium* and *Th. ponticum* subgenomes were used as additional reservoirs of valuable genes, viz. *Th. bessarabicum*, *Th. elongatum*, and *Th. junceiforme*.

Th. bessarabicum (JJ or E^bE^b) showed a high level of salt tolerance (Gorham et al., 1986). To facilitate the gene transfer from *Th. bessarabicum*, hexaploid and octaploid amphiploids ($2n = 4x = 42$, AABBJJ = AABBE^bE^b, or $2n = 8x = 56$, AABBDDJJ) were created (Qi et al., 2010). On their basis, lines with 5A and 5D chromosomes substituted by 5J were produced. Later, a line with translocation T2JS-2BS·2BL from the *Th. bessarabicum* was obtained (Table 2) (Guo J. et al., 2016).

The genomic composition of *Th. elongatum* is currently being clarified using methods of molecular cytogenetics, and di-, tetra-, hexa-, and decaploid forms with the E genome were identified in it (Colmer et al., 2006; Chen S. et al., 2013; Chen C. et al., 2023; Shi et al., 2023). However, when studying decaploids using differentiating GISH subgenomes with *Pseudoroegneria* (St) labeled DNA, two St-like and three E-like subgenomes were revealed (Wang L. et al., 2017; Baker et al., 2020). In this regard, the decaploid forms probably belong to *Th. ponticum*. *Th. elongatum* has tolerance to salinity, drought, water logging, and extreme temperatures (Li Z.S. et al., 2008; Ceoloni et al., 2014; Li X. et al., 2017; Yang Z. et al., 2022). Diploid and tetraploid accessions were used in hybridization, and lines with supplemented, substituted chromosomes and translocations of different size were obtained. Lines with the short arm of *Th. elongatum* chromosome 4Ag carry *Pm* locus for broad-spectrum resistance to powdery mildew (Yang G. et al., 2023).

Tetraploid sea wheatgrass *Th. junceiforme* ($2n = 4x = 28$, $J_1J_1J_2J_2$) is adapted to the coastal areas and is characterized by high tolerance to waterlogging, salinity, manganese toxicity, low nitrogen, and heat stress (Singh D. et al., 2019). At first stage, an amphiploid on the base of the *Th. junceiforme* was obtained, and then the supplemented and introgression lines with translocations were selected. These lines, in addition to abiotic stress tolerance, showed high resistance to *Fusarium* head blight and wheat streak mosaic virus (Singh D. et al., 2019).

In addition to *Thinopyrum* spp., the work has been carried out with genus *Agropyron*. Tetraploid species *A. cristatum* ($2n = 4x = 28$, PPPP) is resistant to powdery mildew, stripe and leaf rusts. Introgression lines with translocations from 2P, 5P, 6P, and 7P chromosomes of *A. cristatum* with valuable genes were produced (Zhang Z. et al., 2017; Jiang et al., 2018). Alien fragments from chromosome 2P and 6P determine a compact plant type with high spike length, spikelet number, and 1,000 grain weight (Zhang Z. et al., 2017; Xu S. et al., 2023). The fragment of a 5P chromosome induced multiple structural rearrangements, including translocations between

chromosomes of different subgenomes. This property can potentially be used as a new tool for inducing wheat–alien chromosome recombination (Li W. et al., 2019).

Currently, information is accumulating that species with relative subgenomes may have similar resistance genes. An accession of diploid *Th. elongatum* has a *Fusarium* head blight resistance gene (*Fhb-7EL*) similar to the designated *Fhb7* gene from *Th. ponticum* (accession el2), and in both accessions the *Fhb* genes were linked with the known *Lr19* gene (Ceoloni et al., 2017; Ma et al., 2018; Kuzmanović et al., 2020). These facts emphasize the need for careful study and comparison of introgressive material obtained on the basis of *Thinopyrum* spp. for determining new genes for resistance to stresses.

Experience of long-term use of tertiary gene pool for defence wheat from diseases

Currently, more than 100 resistance genes to each of the wheat rusts and powdery mildew have been identified, including designated, unknown new genes, and quantitative trait loci (QTLs) (McIntosh et al., 2018). Most of the resistance genes were overcome rather quickly as a result of evolutionary processes in pathogen populations (Kolmer, 2013; Patpour et al., 2022). The use of the tertiary gene pool began in the 1960s, as suitable donor lines were developed. Despite the large amount of introgressive material, a small number of alien genes were intensively used in world breeding programs. This situation was due to the fact that some genes had low protective effect, and others significantly decreased the agronomic properties of cultivars (Friebe et al., 2000). Thus, some of the designated genes transferred from *Thinopyrum* spp. (viz. *Lr29*, *Lr38*, Sr*43*) were not successfully used in breeding due to negative effects on agronomic traits (Zhang W. et al., 2005).

The experience of intensive use of alien genes over several decades has given us knowledge about their effectiveness and impact on pathogen populations. R. Johnson (1983), based on the analysis of crop production experience, proposed the concept of "durable resistance", as resistance that remained effective for a long period when a cultivar is deployed over an extensive area and in environments favourable for the disease (Johnson, 1983). One of the most significant achievements in the use of the tertiary gene pool was cultivar breeding with the 1BL/1RS translocation from rye cv. Petkus, carrying genes for resistance to rusts and powdery mildew (*Lr26*/*Sr31*/*Yr9*/*Pm8*). The wide spread of the *Sr31*-protected cultivars led to suppression of the *P. graminis* f. sp. *tritici* populations worldwide for several decades, until the appearance of race Ug99 in Uganda in 1998 (Singh R.P. et al., 2015). As a consequence of the spread of races of Ug99 group, *Sr31* gene became ineffective in Africa and the Middle East (Singh R.P. et al., 2015; Patpour et al., 2022), but remains effective in the USA, Canada, India, China, and Russia (Brar et al., 2019; Skolotneva et al., 2023; Wu et al., 2023). The history of exploiting cultivars with *Sr31* gene shows that it provided durable wheat resistance to stem rust.

The experience of cultivating varieties created at CIMMYT in various regions of the world has shown that 12 genes turned out to be the most valuable for protection against progressive stem rust. Of these, three genes were obtained from common wheat (*Sr2*, *Sr23* and *SrTmp*), and two genes were transferred from the primary gene pool (*Sr33* and *Sr45*). The remaining *Sr* genes were transferred from the tertiary and secondary gene pools, mainly as part of complex loci, viz. from *Th. ponticum* (*Sr24*/*Lr24*, *Lr19*/*Sr25*), *S. cereale* (*Sr31*/*Lr26*/*Yr9*/*Pm8*, *Sr1RS*^{Amigo}/*Pm17* and *Sr50*), *T. timopheevii* (*Sr36*/*Pm6*), A*e. ventricosa* (*Sr38*/*Lr37*/*Yr17*) (Singh R.P. et al., 2015). Taking into account the high risk of spreading of Ug99 group races, much attention was paid to the effectiveness of known *Sr* genes against it. The genes *Sr25*, *Sr26*, *Sr43*, *Sr61* from *Th. ponticum*, as well as *Sr44* from *Th. intermedium*, are effective against the races of Ug99 group (Zhang J. et al., 2021; Pathotype Tracker, 2023). Before the appearance of Ug99 race, virulence to *Sr24* was rare in *P. graminis* f. sp. *tritici* populations worldwide, but by 2006, virulence appeared in five Ug99 races in Africa (Jin et al., 2008; Bhavani et al., 2019). In Australia, the *Sr24* gene has been effective for about 20 years, and *Sr26* has remained effective for several decades, which can be considered as long-term resistance to stem rust (Zhang J. et al., 2021).

In the 1983–2012 period, about 12.5 thousand varieties and lines of common wheat were created in the world. The genetic material of *Thinopyrum* spp., mainly *Th. ponticum* (93 %), was actively used to protect wheat (Martynov et al., 2016). The distribution of known wheatgrass genes in cultivars varied significantly by region. This may be determined by cultivar adaptation to regional climate, technological requirements for product quality, and pathogen populations. More than half of the North American cultivars had introgressions from *Th. ponticum*, less often they were present in cultivars in Australia (12.6 %), Asia (14.8 %) and South America (8.5 %) (Martynov et al., 2016). In the USA, most winter varieties were protected by *Lr24*/*Sr24* (Kolmer, 2007), and *Lr19*/*Sr25* was present in 12 % of cultivars. In Australia, *Lr24*/*Sr24* were mainly used to protect wheat from rusts (82 %), and *Sr26* and *Lr19*/*Sr25* were used less often. In South Africa and Egypt, about 5 % carried the *Lr24*/*Sr24* genes, and in Russia and China, mainly the *Lr19*/*Sr25* translocation was used (Martynov et al., 2016; Xu X. et al., 2018; Gultyaeva et al., 2021). Over time, the resistance of cultivars with wheatgrass introgressions was overcome by rust fungi in some regions. The *Lr19* gene was overcome in Mexico and India (Huerta-Espino, Singh, 1994; Bhardwaj et al., 2005). *P. triticina* races virulent to *Lr24* appeared in North and South America, and South Africa, where translocation *Sr24*/*Lr24* was used for a long time (Park et al., 2002; Kolmer et al., 2007; Li H., Wang, 2009). On the examples of *P. triticina* populations in the USA, it was shown that the frequencies of virulent to *Lr19* and *Lr24* alleles were higher in the regions where cultivars with complementary genes were mainly cultivated (Kolmer et al., 2007; Kolmer, 2013). In other world regions, where the *Sr24*/*Lr24* translocation has not been used intensively in breeding, cultivars protected with *Sr24* and *Lr24* remain resistant to stem and leaf rusts (Xu X. et al., 2018, Gultyaeva et al., 2021).

Common wheat crops in Russia are an interesting model for evaluating the effectiveness of resistance genes to leaf and stem rusts. The major cropping areas are located in the European (North Caucasian, Central Black Earth, Central, and

the Volga regions), and in the Asian (South Ural and Western Siberia) parts of the country. Different European and Asian populations of *P. triticina* and *P. graminis* f. sp. *tritici* exist on these crops (Gultyaeva et al., 2021; Skolotneva et al., 2023). The Volga region and the Southern Urals are zones of contact between them, due to the spore transfer with air flows (Gultyaeva et al., 2021).

In 1970–2020, regional cultivars with different *Lr* and *Sr* genes from the tertiary gene pool were bred. Some cultivars (from 15 to 30 % in different years) in the Volga region included translocations *Lr19*/*Sr25* and *Lr6Agi*/*Sr6Agi* (Sibikeev et al., 2017; Gultyaeva et al., 2021). *Lr9* and *LrSp* genes (from *Ae. umbellulata*, and *Ae. speltoides*, respectively) were present in South Ural cultivars, and *Lr9* was introduced into West Siberian cultivars. *Lr26*/*Sr31* genes, as well as combinations of less effective *Lr* and *Sr* genes, were present in all regions, and *Sr24*/*Lr24* were rare (less than 1 % of cultivars) (Gultyaeva et al., 2021; Baranova et al., 2023). A long-term study of *P. triticina* populations showed that virulence to *Lr19* prevailed in the population of the Volga region until 2010, but as the spectrum of resistance genes expanded, the frequency of alleles decreased. Virulent to *Lr19* or *Lr9* alleles did not accumulate in *P. triticina* populations if cultivars with different genes were cultivated in the regions. So, in the Central and Northwest regions, close to the Volga region, virulence to *Lr19* and *Lr9* was rare in 2001–2010, and disappeared after 2010 (Gultyaeva et al., 2023). In the South Ural and West Siberian regions, *Lr9* gene was overcome in 2008 (Meshkova et al., 2012), but *Lr19* gene remains effective (Gultyaeva et al., 2021). In all populations, virulence to *Lr24* was extremely rare and virulence to *Lr6Agi* and *LrSp* was completely absent. There were also no pathotypes virulent to the combinations of *Lr19+Lr26* and *Lr9+Lr26* (Gultyaeva et al., 2021).

For a long period, stem rust did not significantly affect wheat crops in most regions of Russia. The first strong disease outbreaks were noted in the Volga region in 2013 and 2014, and in Western Siberia and neighbouring Northern Kazakhstan in 2015 (Shamanin et al., 2016; Sibikeev et al., 2016). At the same time, cultivars with *Sr31* gene were damaged in both regions (Sibikeev et al., 2016; Plotnikova et al., 2022), but virulent races did not belong to Ug99 group (Patpour et al., 2022). In the following years, virulent pathotypes disappeared from populations, and *Sr31* gene remains effective in Russia (Baranova et al., 2023; Skolotneva et al., 2023). By the end of the epidemic of stem rust in Western Siberia in 2015, cultivars and lines with *Sr24*, *Sr25* and *Sr26* genes showed moderate susceptibility, but later their resistance was restored (Plotnikova et al., 2023a). In the Volga region, the lines with *Sr25* were susceptible to stem rust in 2022, whereas those with *Sr24* and *Sr26* genes remained highly resistant (Baranova et al., 2023).

After the appearance of virulent pathotypes to single resistance genes, cultivars began to be protected by gene combinations. Combinations of wheatgrass genes (*Sr24*/*Lr24* or *Lr19*/*Sr25*) with rye *Lr26*/*Sr31* or *T. timopheevii's Sr36*/*Pm6* were highly effective against the rusts in different regions of the world (Park et al., 2002, Martynov et al., 2016; Gultyaeva et al., 2021). In the Volga region, the combinations *Lr19*/*Sr25* + *Lr6Agi*/*Sr6Agi* or *Lr19*/*Sr25* + *Sr22* (from *T. monococcum*) were effective (Sibikeev et al., 2017, 2021). Also, high resistance to leaf and stem rusts was demonstrated by cultivars with combinations of translocations *Sr24*/*Lr24* or *Lr19*/*Sr25* with any APR genes present in complex loci, viz. *Lr34*/*Sr57*/*Yr18*/*Pm38*, *Sr2*/*Lr27*/*Yr30*, *Lr46*/*Sr58*/*Yr29*, *Lr67*/*Sr55*/*Yr46* (Aravindh et al., 2020).

Fitness costs of virulence to genes from tertiary and secondary gene pools and effects of nonhost resistance in introgression wheat

Coevolution of pathogens with host plants is constantly taking place in agroecosystems, aimed at overcoming resistance. Using the example of *P. triticina*, it was shown that new pathotypes regularly appear in populations, but more than half of them occur once, and then disappear (Gultyaeva et al., 2023). To gain a foothold in populations, new forms need to acquire a set of traits that determine their fitness. Parasitic fitness is defined as the relative ability of a parasitic genotype or population to persist over time and contribute to the future gene pool. Fitness depends on genotype viability and reproductive capability (Park et al., 2002). Virulence contributes to the expansion of the range of affected plants, but may have different fitness costs for pathogens. Under favourable conditions, a new pathotype can accumulate additional modifier genes that increase its fitness. However, under stressful conditions, new genes can lead to a decrease in viability and reproduction, which manifests as a fitness penalty for the parasite (Antonovics, Alexander, 1989; Zhan J., McDonald, 2013).

Plants play the role of habitat for parasitic fungi, which is why cultivar genotypes and crop diversity have a great influence on fungal populations. Fitness cost correlates with durable cultivar resistance to fungal diseases. The suppression of *P. graminis* f. sp. *tritici* populations in most world regions after the spread of cultivars protected by the *Sr31* gene during 1960–1990, as well as the disappearance of virulent clones to *Sr31* from Russian populations in the 2020s, indicates that virulence to this gene dramatically reduces pathogen fitness. At the same time, the appearance of Ug99 race demonstrated the possibility of improving fitness when adapting to wheat cultivars with *Sr31* gene in African conditions. The increased frequency of virulent races to *Lr19*, *Lr24*, *Sr24*, and *Sr25* in the regions with a significant proportion of cultivars protected by complementary wheatgrass genes and lower concentration in other regions (Kolmer, 2013; Gultyaeva et al., 2021; Baranova et al., 2023) show that the pathotypes gained a competitive advantage on such cultivars, but had fitness penalties of different degrees on other genotypes. Consequently, virulence to *Lr28* and *LrSp* has not been detected in Russian *P. triticina* populations for decades (Gultyaeva et al., 2021). There was an outbreak of virulence to *Lr47* (frequency up to 70 %) in Western Siberia in 2015, but in the following years virulent clones rapidly disappeared from the population (Plotnikova et al., 2018). It is possible that virulence to *Lr47* has a high fitness penalty for the pathogen.

Pathogenic fungi are not able to exist on species for which they have not been specialized, so-called nonhosts. Nonhost

resistance (NHR) is rarely overcome, so its genetic control and protective mechanisms are of great interest (Niks, 2014). For breeding varieties with durable resistance to diseases, it is considered promising to transfer the defense mechanisms of nonhost species into crops. According to the widely accepted hypothesis formulated in 2010s, nonhost and host resistance is controlled by different genetic systems (PTI and ETI, respectively) (Peng et al., 2018).

When studying the interaction of *P. graminis* f. sp. *tritici* with nonhost *S. cereale* and *Th. ponticum*, it was found that pathogen development was disrupted at an early stage. This was manifested in the disorientation of fungal infection structures on plant surface, and in suppression of appressoria formation, necessary for penetration into the stomata (Plotnikova et al., 2022, 2023a). When infecting wheat lines and cultivars with introgressed rye and wheatgrass genes (*Sr31*, *Sr24*, *Sr25*, and *Sr26*), similar signs of the violation of surface fungal structures was revealed. In addition, the generation of reactive oxygen species (ROS) by stomatal guard cells upon contact with the appressoria was revealed in the lines with these genes. ROS generation led to the death of rust fungus before penetration into plant tissues (Plotnikova et al., 2022, 2023a).

Analogous defence mechanisms were established during the interaction of *P. triticina* with nonhost species, and wheat lines with wheatgrass *Lr19* and *Lr38* genes (Plotnikova, 2008, 2009). Similar ROS generation by stomatal guard cells, called "stomatal immunity", was found when *Arabidopsis thaliana* was infected with non-pathogenic bacteria *Escherichia coli* and *Pseudomonas syringae* pv. *tomato* (Zeng, He, 2010; Melotto, 2017). This indicates that single genes of the secondary and tertiary gene pools may supply defence mechanisms similar to the nonhost ones, which stop infection at the early stages and prevent penetration into the tissues. When virulence occurs, the chemical composition and immunological properties of the fungal cell wall can be changed. Probably, such changes reduce the viability of mutant clones, which leads to a fitness penalty and their disappearance from populations. The appearance of virulence to two genes in the genotype (to *Sr24* + *Sr31*, or *Lr19* + *Lr26*, etc.) leads to the loss/change of a set of important components, which might be lethal for pathotypes. This may explain high cultivar resistance with combinations of wheatgrass and rye translocations to stem and leaf rusts in different world regions.

Thanks to progress in the field of molecular genetics, it has become possible to transfer a set of resistance genes in the form of cassettes (up to five genes) to varieties. The genes controlling PTI-type (non-host) resistance are of particular interest for construction of cultivars with durable resistance to biotrophic pathogens (Liu X. et al., 2021). In this regard, the genes of *Thinopyrum* and related genera, providing protection similar to nonhosts, are promising for creating effective gene cassettes.

Conclusion

Increasing the production of wheat grain is a strategic task to provide food for the growing world population. For sustainable grain production, it is necessary to increase the genetic diversity of cultivars. Species of the secondary and tertiary gene pools with homoeological genomes are of great value for crop protection. *Thinopyrum* and related genera are reservoirs of resistance genes to wheat diseases and abiotic stresses. The most valuable species for breeding are polyploids *Th. ponticum* and *Th. intermedium*. Recently, it has been shown that relative species *Th. elongatum*, *Th. bessarabicum*, *Th. junceiforme*, and *A. cristatum* are also potential donors of valuable traits for wheat improvement. Currently, a large number of introgression lines resistant to a range of wheat diseases (including leaf, stem, and stripe rusts, and powdery mildew, etc.) and tolerant to abiotic factors (such as drought, salinity, and extreme temperature, etc.) have been produced. However, only a small number of introgressions were used in wheat breeding, due to negative effects on agronomic traits. To improve line properties, the work was carried out to reduce the sizes of loci or to use compensating Robertsonian translocations (RobTs).

The experience of long-term cultivation of varieties with the genes from *S. cereale* and *Th. ponticum* has shown that they significantly influence *P. triticina* and *P. graminis* f. sp. *tritici* populations. Obviously, virulent alleles to tall wheatgrass and rye genes reduce the fitness of rust fungi, which leads to partial or complete pathotypes elimination from fungal populations. Cultivars with combinations of wheatgrass and rye translocations showed high resistance to leaf and stem rusts in different regions of the world. *Th. ponticum* and *S. cereale* are nonhosts to *P. graminis* f. sp. *tritici* and *P. triticina*, and their resistance leads to disruption of the development of fungal structures at the plant surface or when trying to penetrate into the stomata. The introgressed *Sr24*, *Sr25*, *Sr26*, *Lr19*, *Lr38*, and *Sr31* genes control manifestations of protective mechanisms similar to nonhost resistance. Such action makes these genes (and the genes with analogous action) promising for engineering crops with the help of molecular technologies.

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Distribution and species composition of potato viruses in the Novosibirsk region

V.S. Maslennikova[1, 2], M.B. Pykhtina[1], K.A. Tabanyukhov[1, 2], E.V. Shelikhova[1, 2], K.I. Mosalev[1], A.V. Katokhin[1], A.A. Bondar[3], A.B. Beklemishev[1], M.I. Voevoda[1]

[1] Federal Research Center of Fundamental and Translational Medicine, Novosibirsk, Russia
[2] Novosibirsk State Agrarian University, Novosibirsk, Russia
[3] Institute of Chemical Biology and Fundamental Medicine of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
pykhtina_maria@mail.ru

Abstract. Among the many diseases that affect potato plants, viral infections are the most common and cause significant damage to farms, affecting both the yield and quality of potatoes. In this regard, an important condition for preserving the potato seed fund in Russia is systematic monitoring and early highly specific detection of potato viral infections. The purpose of the work is to study samples of potato varieties collected in the Novosibirsk region for the presence of viral infections using RT-PCR. 130 potato plants from three districts of the Novosibirsk region (NR) were studied. As a result of monitoring, the following viruses were identified: PVY (potato virus Y), PVS (potato virus S), PVM (potato virus M) and PVX (potato virus X). The quarantine pathogen potato spindle tuber viroid (PSTVd) was not detected in any of the samples analyzed. The maximum frequency of occurrence in the region was noted for three viruses: PVY, PVM and PVS. A significant proportion of the samples were mixed viral infections: the occurrence of the combination of infection PVY + PVM in plants was 25.0 %, and PVY + PVS, 22.6 %. To develop methods for determining the strain affiliation of the studied samples, the nucleotide sequences of the capsid protein genes of 10 Y-virus isolates were sequenced. Phylogenetic analysis of the studied sequences of NR isolates was carried out with a set of sequences of reference strains 261-4, Eu-N, N:O, NE-11, NTNa, NTNb, N-Wi, O, O5, SYR_I, SYR_II and SYR_III retrieved from GenBank. As a result of phylogenetic analysis, it was established that NR viral samples fell into two groups of strains: group 1, which also includes isolates of the reference strains 261-4/SYR_III, and group 2, NTNa. The obtained results of the strain affiliation of NR samples lay the basis for the development of DNA and immunodiagnostic systems for identifying PVY circulating in NR, as well as for elucidating the source and routes of entry of specific virus strains.
Key words: *Solanum tuberosum*; viral infections; RT-PCR; potato Y virus; phylogenetic analysis.

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Распространенность и видовой состав вирусов картофеля в Новосибирской области

В.С. Масленникова[1, 2], М.Б. Пыхтина[1], К.А. Табанюхов[1, 2], Е.В. Шелихова[1, 2], К.И. Мосалев[1], А.В. Катохин[1], А.А. Бондарь[3], А.Б. Беклемишев[1], М.И. Воевода[1]

[1] Федеральный исследовательский центр фундаментальной и трансляционной медицины, Новосибирск, Россия
[2] Новосибирский государственный аграрный университет, Новосибирск, Россия
[3] Институт химической биологии и фундаментальной медицины Сибирского отделения Российской академии наук, Новосибирск, Россия
pykhtina_maria@mail.ru

Аннотация. Среди множества болезней, поражающих растения картофеля, именно вирусные инфекции являются наиболее распространенными и наносят значительный ущерб хозяйствам, влияя как на урожайность, так и на качество картофеля. В связи с этим важное условие сохранения семенного фонда картофеля в России – систематический мониторинг и раннее высокоспецифичное обнаружение вирусных инфекций картофеля. Целью работы было исследование образцов сортов картофеля, собранных на территории Новосибирской области (НСО), на наличие вирусных инфекций методом ОТ-ПЦР. Изучено 130 растений картофеля из четырех районов Новосибирской области. В результате мониторинга обнаружены следующие вирусы: PVY (potato

virus Y), PVS (potato virus S), PVM (potato virus M) и PVX (potato virus X). Ни в одном из анализируемых образцов не найден карантинный объект – вироид веретеновидности клубней картофеля (potato spindle tuber viroid – PSTVd). Максимальная частота встречаемости в районах области была отмечена для трех вирусов – PVY, PVM и PVS. Смешанные вирусные инфекции составили заметную долю образцов: встречаемость комбинации инфекции PVY + PVM в растениях составляла 25.0 %, PVY + PVS – 22.6 %. Для отработки методов выяснения штаммовой принадлежности изучаемых образцов проведено секвенирование нуклеотидных последовательностей капсидных белков 10 изолятов Y-вируса. С просеквенированными последовательностями был осуществлен филогенетический анализ совместно с набором последовательностей референсных штаммов 261-4, Eu-N, N:O, NE-11, NTNa, NTNb, N-Wi, O, O5, SYR_I, SYR_II, SYR_III, взятых в GenBank. В результате филогенетического анализа установлено, что образцы из НСО распределились в две группы штаммов: группа 1, включающая также изоляты референсных штаммов 261-4/SYR_III, и группа 2 – NTNa. Полученные результаты штаммовой принадлежности образцов из НСО закладывают основу для разработки ДНК- и иммуннодиагностических систем для выявления PVY, циркулирующих в НСО, а также для выяснения источника и путей проникновения конкретных штаммов вируса.

Ключевые слова: *Solanum tuberosum*; вирусные инфекции; ОТ-ПЦР; Y-вирус картофеля; филогенетический анализ.

Introduction

The Novosibirsk region is a favorable region for potato growing (Batov, Gureeva, 2023). The area of its cultivation in the industrial potato growing sector (data on agricultural organizations and peasant farms, excluding households) of the Novosibirsk region in 2023 amounted to 3.8 thousand hectares, which is 6.2 % (0.2 thousand hectares) more than in 2022. At the same time, the total potato harvest in the industrial potato growing sector of the Novosibirsk region amounted to 74.9 thousand tons, which is 12.9 % (8.5 thousand tons) more than in 2022. The top 10 districts in the Novosibirsk region by the size of the harvested potato area in 2023 included: Novosibirsk (36.8 % of the total area), Ordynsky (25.6 %), Moshkovsky (18.6 %), Karasuksky (5.2 %), Toguchinsky (4.4 %), Cherepanovsky (3.3 %), Suzunsky (1.8 %), Iskitimsky (1.7 %), Kochenevsky (1.3 %), Bagansky (0.4 %). The remaining districts accounted for a total of 1.0 % (https://ab-centre.ru/news/rynok-kartofelya-novosibirskoy-oblasti---klyuchevye-tendencii).

According to the Federal State Statistics Service, the average potato yield in Russia is about 16 t/ha (https://rosstat.gov.ru/enterprise_economy), in the Novosibirsk region it is 22.5 t/ha (Batov, Gureeva, 2023), while the maximum productivity of individual varieties of this crop can reach 400 t/ha (State Register of Selection Achievements…, https://gossortrf.ru/). Decrease in yield mostly depends on the influence of various external factors, including the prevalence of a large number of viral pathogens.

Currently, 40 phytopathogenic potato viruses have been identified in different countries and regions (Hameed et al., 2014; Onditi et al., 2021). The most important of them, which have become ubiquitous wherever potatoes are grown, are potato leaf roll virus (PLRV), potato virus Y (PVY), potato virus X (PVX), potato virus S (PVS), potato virus M (PVM). Each of these pathogens is capable of causing yield losses of 10 to 60 %, and, in case of mixed virus infection, losses can be even higher (Byarugaba et al., 2020).

PVY is the fifth most important plant virus in the world (Scholthof et al., 2011) and causes the greatest economic losses in potato production, but also affects other common crops such as tomato, pepper, and tobacco (Kerlan, Moury, 2008; Lacomme et al., 2017). The PVY genome is highly variable and is susceptible to recombination. PVY exists as a complex of strains that can be defined based on hypersensitivity reactions (HR) to three known potato *N* genes (Jones, 1990; Chikh-Ali et al., 2014) or based on genome sequences and recombination patterns (Karasev, Gray, 2013; Green et al., 2017). Currently, fourteen PVY strains have been identified (Karasev, Gray, 2013; Green et al., 2017), including five non-recombinants (PVYO, PVYEu-N, PVYNA-N, PVYC, and PVYO-O5) and nine recombinants (PVY-N:O, PVY-N-Wi, PVY-NTNa, PVY-NTNb, PVY-NE11, PVY-E, PVY-SYR-I, -II, and -III) (Chikh-Ali et al., 2016a, b; Green et al., 2017). Fourteen additional recombinants and genome variants have also been reported (Green et al., 2018).

Since diseases caused by potato viruses are incurable in field conditions, early detection of these pathogens and determination of their species composition is an actual task for agriculture and is included in the subprogram “Development of potato breeding and seed production in the Russian Federation” of the Federal Scientific and Technical Program for the Development of Agriculture for 2017–2025.

Currently, there are three main methods for diagnosing the virus in potato tubers: real-time RT-PCR, enzyme-linked immunosorbent assay (ELISA), and immunochromatographic assay.

Previously, studies of virus load on potato agrocenoses were conducted in some regions of the Russian Federation. In 2016, in the Astrakhan region, a high incidence of the Y virus was recorded on all plantings of early reproductive potatoes, with the exception of the Krona variety, especially on the Impala (65–95 %), Red Scarlett (85 %) and Courage (60 %) varieties. In 2017, on the Impala variety, while a high incidence of PVY was maintained (60 % of plants), significant damage (50 % of plants) by PVS and PVM was observed (Fominykh et al., 2017). The frequency of PVS and PVM in the Republic of Bashkortostan was 87 % and 78 %, respectively, PVX – 12 %, PVY – 28 %. Up to 61.6 % of tubers were infected with two viruses (PVS+PVY, PVS+PVX and PVM+PVY) and 2.8 % of samples were infected with a combination of three viruses. Only 6.9 % of the studied samples were virus-free (Khairullin et al., 2021).

Given the high incidence of viral infections in potato plants in various regions of Russia, early and accurate diagnostics of

viral infections as well as study of the genetic polymorphism of individual strains of the most common virus species are extremely important. After the introduction of PCR diagnostic methods, abundant data on the genetic diversity of PVY strains began to appear, and it became possible to conduct more detailed studies aimed at identifying the sources and routes of spread of potato viruses. For example, based on the results of monitoring the occurrence of viruses in samples of 4 potato varieties (Red Scarlett, Silvana, Labella, Nevsky) using the RT-PCR method, it was found that 100 % of plants were infected with the X virus and 26.3 % were infected with the Y virus (Grigoryan, Tkachenko, 2019), and the infection of potatoes with the Y virus in the Perm' region in 2019 was 100 % (Pechenkina, Boronnikova, 2020).

The studies by A.M. Malko et al. (2017) showed a high incidence of PVY in the Samara, Tver', and Leningrad regions (33.3, 29.2, and 25.7 %, respectively), that of PVS in the Samara and Irkutsk regions (66.7 and 30.5 %, respectively), and that of PVM in the Tver', Samara, and Nizhny Novgorod regions (25.0, 22.2, and 19.4 %, respectively) (Malko et al., 2017). Diagnostics of potato viral diseases using real-time PCR, conducted in 2019 in the Saratov region, detected PVY in two potato varieties, in the absence of visual plant lesions.

Since 2015, the Federal Research Center for Potatoes named after A.G. Lorkh has been studying the serological and phytopathological characteristics of PVY isolates from various regions of the Russian Federation, including the Novosibirsk Region. Out of the seven identified isolates with PVY monoinfection in the material from the Novosibirsk Region, five isolates exhibited serological and phytopathological properties of $PVY^{O/C}$ (common strain and acropetal necrosis strain) (Uskov et al., 2022).

The aim of this work was to study the species composition of potato viruses of different varieties and categories and the incidence of plants in farms of the Novosibirsk region using molecular genetic methods to determine their prevalence in seed tubers, as well as to study the strain composition of individual PVY isolates.

Materials and methods

The work was completed in 2023. The studies were conducted on 130 *S. tuberosum* potato plants from Iskitimsky (varieties Gala (RS1), Red Scarlett (E), Rosara (RS1)), Ordynsky (varieties Gala (RS1), Lady Claire (RS1), Rosara (RS1)), Kochenevsky (varieties Zlatka (SE), Rosara (RS1)) and Novosibirsk (varieties Gala (RS1), Red Scarlett (RS1)) districts of the Novosibirsk region (Table 1).

The samples were supplied by farms from the specified regions under an agreement with the Federal State Budgetary Institution "Rosselkhozcentr" in the Novosibirsk Region, which were selected in accordance with GOST 33996-2016. Ten samples were analyzed, the samples from the Iskitimsky district contained 20 tubers each, while the samples from the Ordynsky, Kochenevsky and Novosibirsk districts contained 10 tubers each. Potato tubers of each variety were cultivated in plastic pots (0.7 l) in boxes at a temperature of +24 °C ± 1 °C and a photoperiod of 16/8 hours: light/dark. Leaf samples for determining the viral load were collected four weeks after planting from the upper, middle and lower tiers of plants. Among the studied samples, four varieties (Rosara, Lady Claire, Gala, Red Scarlett) are varieties of foreign selection, and one variety (Zlatka) is of domestic selection. Isolation of viral RNA from the collected potato leaves was performed using the "PhytoSorb" kit manufactured by SYNTOL (Russia) in accordance with the manufacturer's recommendations. RNA analysis was performed on a Rotor-Gene Q amplifier (Qiagen, Germany). The presence of viruses in potato leaf samples was determined using a reagent kit (by SYNTOL) PV-005 (PVX, PVY, PVM, PLRV, PVA, PVS and PSTVd).

Sample preparation for DNA sequencing. Individual Y-positive isolates (10 samples) were selected for cDNA synthesis and subsequent sequencing of the capsid protein gene region. Reverse transcription was performed using the RT M-MuLV-RH reagent kit (Biolabmix, Russia) according to the manufacturer's protocol: 2–5 μg of total RNA was taken per reaction and primers (473-F: 5′-CAAATGACACAATCG ATGCA-3′; 474-R 5′-CATGTTCTTGACTCCAAGTAGA GTATG-3′) were designed for synthesis of the first and then

Table 1. Analyzed potato material by districts of the Novosibirsk region

District	Variety, ripening period	Number of analyzed tubers, pcs	Reproduction
Iskitimsky	Gala, mid-early ripening	20	RS1
	Red Scarlett, early ripening	20	E
	Rosara, early ripening	20	RS1
Novosibirsk	Gala, mid-early ripening	10	RS1
	Red Scarlett, early ripening	10	RS1
Kochenevsky	Zlatka, mid-season ripening	10	SE
	Rosara, early ripening	10	RS1
Ordynsky	Gala, mid-early ripening	10	RS1
	Lady Claire, early ripening	10	RS1
	Rosara, early ripening	10	RS1

the second strand of cDNA at the PVY genomic RNA site encoding the capsid protein of the virus. The primers were selected based on comparison of the nucleotide sequences of the envelope protein gene of known Y virus isolates represented in GenBank.

The synthesized DNA was further used for PCR amplification of the coding region of the PVY capsid protein gene of the tested virus isolates. PCR was performed in a reaction mixture containing the above-mentioned primers 473-F and 474-R. The mixture was heated for 5 min at 70 °C and transferred to an ice bath for 2 min; then the mixture of the remaining reagents (RNA-dependent DNA polymerase, RT buffer, deoxynucleotide triphosphates) was incubated for 10 min at room temperature; then it was transferred to a thermostat at 42 °C for 2 h; at the end, the reaction was stopped by heating for 15 min at 70 °C. Quantitative PCR with real-time detection was performed using "BioMaster HS-qPCR SYBR Blue(2×)" by Biolabmix. PCR was performed in a CFX96 Touch amplifier (2014, Bio-Rad Laboratories, USA) according to the following amplification program: DNA denaturation at 95 °C for 1 min, followed by 40 PCR cycles (DNA denaturation at 95 °C for 20 s, primer annealing at 55 °C for 15 s, DNA chain elongation at 72 °C for 30 s). Amplification products were separated by gel electrophoresis in 0.8 % agarose gel containing 0.00005 % EtBr.

Sequencing of amplicons of the capsid protein gene of PVY isolates. The amplicons ~800 bp in size encoding the capsid protein of potato virus Y (PVY) were purified from PCR components of the reaction mixture by sorption on SpeedBead magnetic particles (GE Healthcare, USA) in the presence of 7 % PEG-8000. After washing three times with 80 % ethanol, amplicons were eluted with MiliQ water. For the Sanger sequencing reaction, 0.5 pmol of amplicon, 20 pmol of one of the primers (473_F_coat-Y-vir or 474_R_coat-Y-vir), 2 µl of BigDye v.3.1 reagent, 8 µl of 5X sequencing buffer (Nimagen, USA), 8 µl of 5M betaine and MiliQ water were used up to a total reaction volume of 40 µl. The temperature profile of the Sanger reaction consisted of: denaturation at 96 °C for 3 min, followed by 70 cycles (melting at 96 °C for 25 s; annealing at 40 °C for 10 s; elongation at 60 °C for 3 min) with a final warm-up at 98 °C for 5 min and storage until purification at 4 °C. The Sanger reactions were then purified from unreacted BigDye by gel filtration in tablet format microcolumns through Sephadex G-50 semisolid column (GE Healthcare, USA) by centrifugation at 1,700 g for 4 min. The products of the Sanger reaction were analyzed on an ABI 3500XL automated gene analyzer (Applied Biosystems, USA) at the Genomics CDC (ICBFM SB RAS). Nucleotide sequences of the studied amplicons were used for analysis by alignment and comparison with the GenBank database (NCBI, USA).

Comparison of nucleotide sequences of the covering capsid protein of the Y virus. For phylogenetic analysis, the nucleotide sequences of the capsid gene of PVY isolates from the Novosibirsk region were compared using the MAFFT service (https://www.ebi.ac.uk/Tools/msa/mafft/) with the corresponding reference sequences provided in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Analyses were performed with MEGAX software (Kumar et al., 2018) using maximum likelihood (ML) algorithm. Nucleotide sequence-based phylogram construction was performed considering all codon positions using evolutionary models of substitutions specified by the MEGAX>Models module: TN92(G+I) (Tamura–Nei). Phylogram construction based on amino acid sequences was performed using the JTT(G+I) module (Jones–Taylor–Thorton). The following named sequences were used as reference sequences for the cluster of strains: "261-4": KY848023, AM113988, JF927755; "Eu-N": KY847988, KY847986, JQ969036; "N:O": KY847974, KY848018, AY884985, Z70238, AJ584851; "NE-11": JQ971975, HQ912867; "NTNa": AJ890344, M95491_i, AJ890345, AY884982; "NTNb": AJ890343; "N-Wi": KY847961, AJ890350, JQ924286, JN034046, AJ890349, KY847996; "O": HQ912865, FJ643479, EF026074, AJ585196, JX424837; "O5": FJ643477, U09509, HM367076, HQ912909, KY848035; "SYR_I": GQ200836; "SYR_II": AJ889867; "SYR_III": AB461454. The bootstrap method (500 iterations) was used to determine the stability of the dendrograms.

Statistics. Virus occurrence was assessed using the χ^2 test with Yates' correction.

Results

The highest frequency of occurrence in the districts of Novosibirsk region was noted for three viruses – PVY, PVM and PVS (Table 2). PVY was found in all the studied districts and affected all potato varieties, unlike the M and S viruses. The distribution of viruses across the districts of the Novosibirsk region was uneven (Table 3).

The highest level of PVY infection was detected in the Novosibirsk district, where its prevalence on the Gala variety reached 100 %. Potato leafroll virus was detected on the same variety (20 %). PVS was found in all districts of the region, but the highest prevalence (30–100 %) was detected in the Ordynsky and Kochenevsky districts. Potato virus X was found in the Iskitimsky and Ordynsky districts (40–50 %). It should also be noted that due to the widespread cultivation of foreign varieties in our region, virus M was highly prevalent (40–100 %). Mid-early varieties (Gala, Zlatka) were more often affected by PVM than early-ripening ones. The highest viral load (PVX, PVY, PVM, PVA, PVS) was detected on the Rosara variety of the Ordynsky district. Potato spindle tuber viroid (quarantine object) was absent from all tested samples.

Mixed viral infections made up a significant proportion of the samples: the incidence of the PVY+PVM infection combination in plants was 25.0 %, PVY+PVS – 22.6 %, PVY+PVX – 3.8 % (Table 4). At the same time, the prevalence of "monoinfection" of any virus (PVS, PVM, PVX, PVY) was 19.4 %, and the number of plants in which there were no viruses was less than 1 %. Three viruses in the PVS+PVM+PVY combination were detected in 15.37 % of the samples, and four viruses were detected in 1.8 % (PVS+PVM+PVX+PVY).

To determine the strain identification of the studied samples from the Novosibirsk region, amplified fragments of the PVY genome corresponding to the mature peptide of the capsid protein were sequenced and analyzed by phylogenetic methods using reference sequences from GenBank, described in detail in the article (Green et al., 2017, 2018). The registration numbers of the reference sequences are given in the

Table 2. Prevalence of potato viruses by districts of the region, %

Variety	PVX	PVY	PVM	PLRV	PVA	PVS	PSTVd
Iskitimsky district							
Gala	50	30	75	–	–	55	–
Red Scarlett	–	90	80	–	–	–	–
Rosara	–	70	–	–	–	90	–
Kochenevsky district							
Zlatka	–	60	100	–	–	100	–
Rosara	–	50	–	–	–	–	–
Novosibirsk district							
Red Scarlett	–	60	–	–	–	30	–
Gala	–	100	100	20	–	80	–
Ordynsky district							
Rosara	40	60	40	–	20	100	–
Gala	–	20	–	–	–	20	–
Lady Claire	–	60	–	–	–	–	–

Table 3. Cases of potato virus infection in different districts of the Novosibirsk Region

District	Variety	Number of plants, pcs	Infected plants, pcs							χ^2	p
			PVX	PVY	PSTVd	PVM	PLRV	PVS	PVA		
Iskitimsky	Red Scarlett	20	0	18	0	16	0	0	0		
	Gala	20	10	6	0	15	0	11	0		
	Rosara	20	0	14	0	0	0	18	0		
	Total by varieties		10	38	0	31	0	29	0	112.067	$7.52 \cdot 10^{-22}$
Novosibirsk	Red Scarlett	10	0	6	0	0	0	3	0		
	Gala	10	0	10	0	10	2	8	0		
	Total by varieties		0	16	0	10	2	11	0	53.2	$1.07 \cdot 10^{-9}$
Kochenevsky	Rosara	10	0	5	0	0	0	0	0		
	Zlatka	10	0	6	0	10	0	10	0		
	Total by varieties		0	11	0	10	0	10	0	37.94	$1.16 \cdot 10^{-6}$
Ordynsky	Rosara	10	4	6	0	4	0	10	2		
	Gala	10	0	2	0	0	0	2	0		
	Lady Claire	10	0	6	0	0	0	0	0		
	Total by varieties		4	14	0	4	0	12	2	38.2	$1.03 \cdot 10^{-6}$

Note. The hypothesis about the prevalence of certain potato viruses in the districts of the region was tested using the χ^2 criterion with Yates' correction. *P* values are defined as $p = 0.000$.

"Materials and methods" section. The dendrograms obtained in the MEGAX program based on nucleotide and amino acid sequences made it possible to visualize the distribution of the reference strains used.

The most compact group was formed by the strains of the O5 cluster, representing samples from North America with the eponymous serotype O5. This cluster was used as a proxy "outgroup" in constructing dendrograms to determine the approximate direction of evolution of PVY genetic diversity. The remaining clusters of strains were grouped less clearly. This can be explained by the fact that when constructing monolocus dendrograms, as in our case, there is no way to

Table 4. Frequency of occurrence of potato viruses

Combination of viruses	Frequency of occurrence, %
No infection	0.88
Monoinfection:	
PVS	6.85
PVM	1.25
PVY	11.25
PVX	0
Two viruses:	
PVS+PVM	6.25
PVS+PVY	22.60
PVM+PVY	25.00
PVS+PVX	3.75
PVX+PVY	3.75
Three viruses:	
PVS+PVM+PVY	15.37
PVS+PVY+PVA	1.25
Four viruses:	
PVS+PVM+PVX+PVY	1.80

reflect the consequences of recombination events. Such events are known to occur all the time as viruses adapt to overcome the defenses of infected host plants and spread to new plants.

As is shown in the Figure, the samples from the Novosibirsk region were distributed into two groups of strains: group 1, including samples NSO01-05 and NSO08-09, is combined with the strains of the clusters "261-4" and "SYR_III", and group 2, including samples NSO06-07 and NSO10, is combined with the strains of the cluster "NTNa".

Comparison of the topologies of the nucleotide and amino acid dendrograms also allows to make the expected conclusion that a significant part of the nucleotide diversity of viral sequences does not manifest itself at the level of encoded peptides. It is evident that the Novosibirsk region samples of the first group are identical to each other at the amino acid level and will probably have the same immunochemical properties in the case of using the epitopes of the mature capsid protein as a serological test. The same can be said about the Novosibirsk region samples of the second group. It can be expected that, in the presence of common epitopes, some of them will still differ so much between representatives of the two groups under study that it will be possible to develop differential serological tests.

Discussion

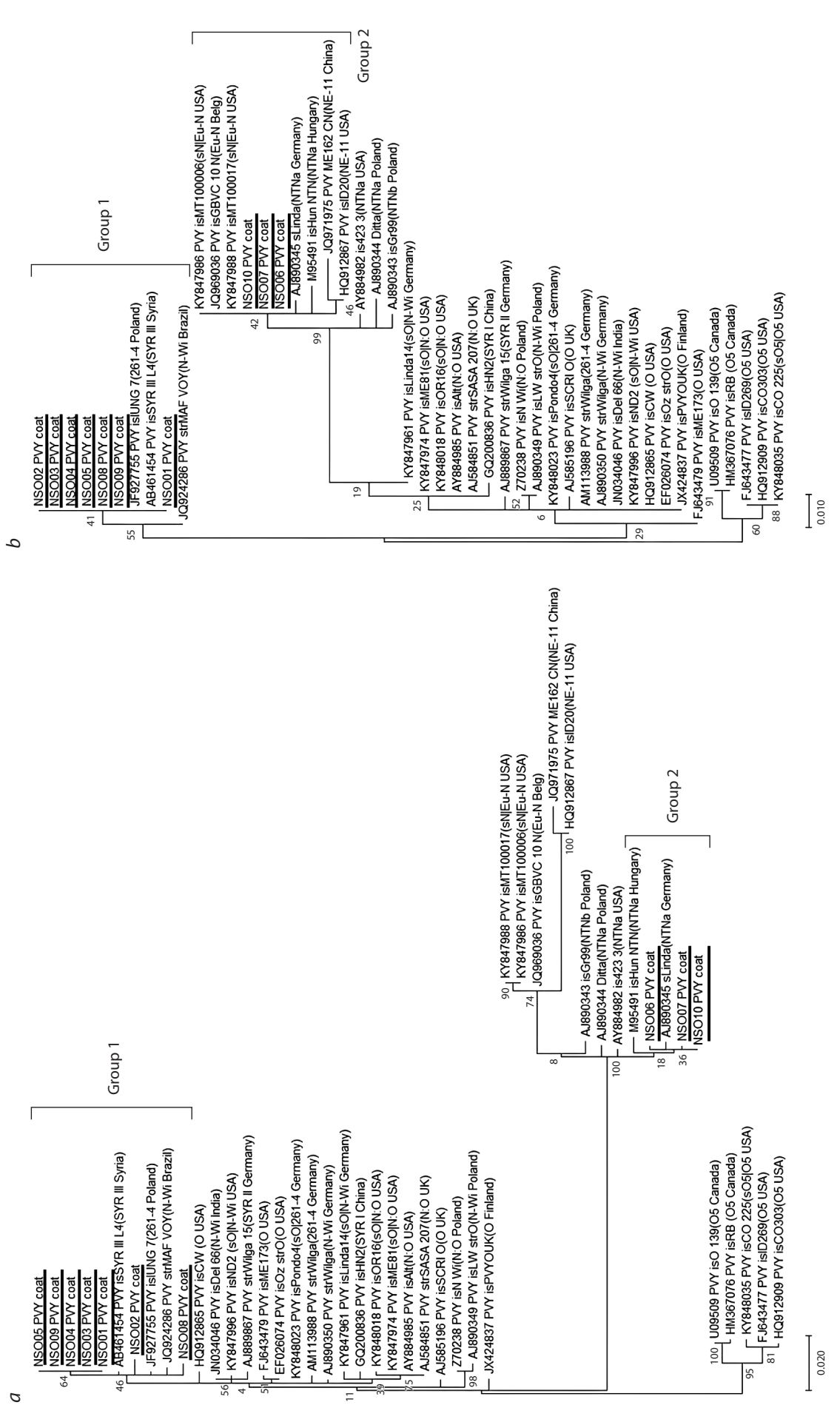
Potato viral infections lead to a significant reduction in its yield, and therefore monitoring of the seed material contamination is a necessary measure for stable and sustainable production of this crop.

In this work, the RT-PCR method was used to monitor viral infections of seed potatoes in the Novosibirsk region, which revealed a high viral load. Among the analyzed samples, no differences in the distribution of viruses associated with varietal resistance and/or reproduction were found. Based on the analysis of the prevalence of viral infections, it was found that plants are most often infected with PVY, PVS and PVM viruses, which were found almost everywhere in the studied areas of the region with a frequency of 30–100 %. Unlike most other potato viruses, PVY is expanding its geographic distribution and causes economic damage to potato crops not only in Russia, but throughout the world (Byarugaba et al., 2020; Kreuze et al., 2020). Mixed viral infection including PVY is the most common (Kerlan, Moury, 2008), since most potato varieties are not resistant to it (Ahmadvand et al., 2012).

Potato plants grown in the Novosibirsk region were typically affected by two viruses (61.35 % of samples), of which PVM+PVY viruses were most common (25.0 %). The presence of three or four viruses simultaneously was detected in 16.62 % and 1.8 % of samples, respectively. Plants affected by viruses were stunted, leaf blades were underdeveloped. Rapid and premature growth of axillary buds was observed. Wrinkling and folding of leaves, their deep venation, chlorosis, and marginal necrosis were noted. This result confirms the results of other scientists (Khairullin et al., 2021), which showed that potatoes can be simultaneously infected with more than four viruses, including the most economically important viruses. The widespread distribution of viruses on potatoes is facilitated by the high infestation of fields with perennial weeds that act as reservoirs of viral infection (Szabó et al., 2020), and by the huge species diversity and the high number of carriers (Danci et al., 2009; Fox et al., 2017).

Since potato viral diseases are incurable, preventive measures aimed at using varieties resistant to viral infections and uninfected seed material are of great importance. These preventive measures require systematic early detection of viral infections, the absence of which has led to mass infection of potatoes with phytopathogens in Russia, including seed material. Therefore, the creation of highly sensitive, early and field-usable diagnostics of potato viral infections is an urgent task.

PVY is considered one of the most significant viruses affecting both potatoes and other economically important species of nightshades (pepper, tomato, tobacco). Since, according to the results of our studies, the highest percentage of samples were infected with this type of virus, it was of interest to determine the nucleotide sequences of the capsid protein gene of the studied PVY isolates from the Novosibirsk region in order to determine the level of conservatism of these proteins for the subsequent creation of an immunochromatographic test system that is highly specific for the Siberian region. Phylogenetic analysis of the obtained samples revealed two groups of PVY strains among them: a group including the strains "261-4/SYR_III" and group 2 – "NTNa". PVY is becoming increasingly widespread throughout the world, mainly due to the increase in the incidence of recombinant forms of the virus, such as PVYNWi and PVYNTN. These strains are highly virulent and have mild symptoms, which complicates their detection in seed potatoes.



Phylogenetic analysis of PVY isolates from Novosibirsk region together with samples submitted to GenBank.

Strain cluster designations and country of sample identification are indicated in parentheses. Serological classes are indicated for some samples, e. g. "sO" before the vertical dash. Samples from Novosibirsk region are underlined with a black dash. *a* – ML-dendrogram based on nucleotide sequences. *b* – ML-dendrogram based on amino acid sequences.

Our data are consistent with the data of other authors who studied the strains of Y virus isolates in the territory of the Russian Federation. A.I. Uskov et al. (2016), when studying the strain composition of the Y virus of potato, common in the territory of the Russian Federation in 2015–2016, identified the ordinary strain PVYO in one variety sample, the tuber ring necrosis strain PVYNTN in 19, the recombinant strain PVYN:O in 36, and two strains PVYNTN and PVYN:O simultaneously in 53 variety samples. Based on a comparative analysis of the marker sequence of the 5′-untranslated region NTR locus, A.A. Stakheev et al. (2023) determined that potato virus Y isolates distributed in various territories of the Russian Federation belonged mainly to the necrotic and recombinant groups of strains, with the exception of a single isolate occupying an intermediate position between these two groups.

Determination of the PVY strain identification not only is of great importance in terms of improving strategies to combat this virus, but also has great diagnostic value. From a comparison of the topologies of the nucleotide and amino acid dendrograms, it follows that both groups of samples from the Novosibirsk region that we identified do not show intragroup differences at the amino acid level, which may indicate serological similarity of samples in a group and the prospects for developing differential serological diagnostics for samples from different groups.

Conclusion

Thus, when developing DNA and immunodiagnostic systems for detecting PVY circulating in the Novosibirsk region, it is possible to use primarily the genetic variations of the virus of these strain clusters.

The obtained results of the strain identification of samples from the Novosibirsk region make the foundation for identifying the source and routes of penetration of specific strains of the virus, as well as for assessing the phytopathogenic risks for potato varieties used in the Novosibirsk region.

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Earthworm (Oligochaeta, Lumbricidae) intraspecific genetic variation and polyploidy

S.V. Shekhovtsov [1, 2], Ye.A. Derzhinsky [3], E.V. Golovanova [4]

[1] Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
[2] Institute of Biological Problems of the North of the Far-Eastern Branch of the Russian Academy of Sciences, Magadan, Russia
[3] Vitebsk State University named after P.M. Masherov, Vitebsk, Belarus
[4] Omsk State Pedagogical University, Omsk, Russia

shekhovtsov@bionet.nsc.ru

Abstract. Earthworms are known for their intricate systematics and a diverse range of reproduction modes, including outcrossing, self-fertilization, parthenogenesis, and some other modes, which can occasionally coexist in a single species. Moreover, they exhibit considerable intraspecific karyotype diversity, with ploidy levels varying from di- to decaploid, as well as high genetic variation. In some cases, a single species may exhibit significant morphological variation, contain several races of different ploidy, and harbor multiple genetic lineages that display significant divergence in both nuclear and mitochondrial DNA. However, the relationship between ploidy races and genetic lineages in earthworms remains largely unexplored. To address this question, we conducted a comprehensive review of available data on earthworm genetic diversity and karyotypes. Our analysis revealed that in many cases, a single genetic lineage appears to encompass populations with different ploidy levels, indicating recent polyploidization. On the other hand, some other cases like *Octolasion tyrtaeum* and *Dendrobaena schmidti*/*D. tellermanica* demonstrate pronounced genetic boundaries between ploidy races, implying that they diverged long ago. Certain cases like the *Eisenia nordenskioldi* complex represent a complex picture with ancient divergence between lineages and both ancient and recent polyploidization. The comparison of phylogenetic and cytological data suggests that some ploidy races have arisen independently multiple times, which supports the early findings by T.S. Vsevolodova-Perel and T.V. Malinina. The key to such a complex picture is probably the plasticity of reproductive modes in earthworms, which encompass diverse modes of sexual and asexual reproduction; also, it has been demonstrated that even high-ploidy forms can retain amphimixis.

Key words: genetic lineages; karyotypes; phylogeny; species divergence.

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Дождевые черви (Oligochaeta, Lumbricidae): соответствие между внутривидовым генетическим разнообразием и плоидностью

С.В. Шеховцов [1, 2], Е.А. Держинский [3], Е.В. Голованова [4]

[1] Федеральный исследовательский центр Институт цитологии и генетики Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Институт биологических проблем Севера Дальневосточного отделения Российской академии наук, Магадан, Россия
[3] Витебский государственный университет имени П.М. Машерова, Витебск, Беларусь
[4] Омский государственный педагогический университет, Омск, Россия

shekhovtsov@bionet.nsc.ru

Аннотация. Дождевые черви известны своей сложной систематикой и разнообразным набором типов размножения, включая амфимиксис, самооплодотворение, партеногенез и некоторые другие способы, которые иногда могут сосуществовать в пределах одного вида. Более того, они демонстрируют значительное внутривидовое разнообразие кариотипов с уровнями плоидности от ди- до декаплоидных и выше, а также высокую генетическую изменчивость. В некоторых случаях один вид может сочетать большую морфологическую изменчивость, несколько рас с разной плоидностью и несколько филогенетических линий со значительными

различиями как по ядерной, так и по митохондриальной ДНК. При этом соответствие между расами различной плоидности и генетическими линиями дождевых червей остается в значительной степени неисследованным. В связи с этим мы провели всесторонний обзор имеющихся данных о генетическом разнообразии и кариотипах дождевых червей. Наш анализ показал, что во многих случаях одна генетическая линия включает в себя популяции с разными уровнями плоидности, что указывает на недавнюю полиплоидизацию. С другой стороны, в некоторых случаях, как, например, *Octolasion tyrtaeum* и *Dendrobaena schmidti*/*D. tellermanica*, имеют место выраженные генетические границы между расами, что означает давнюю дивергенцию между ними. Некоторые таксоны, такие как комплекс *Eisenia nordenskioldi*, представляют собой множество давно дивергировавших филогенетических линий со сложными родственными отношениями между ними и как древней, так и недавней полиплоидией. Сопоставление филогенетических и цитологических данных позволяет предположить, что некоторые полиплоидные расы независимо возникали несколько раз, что подтверждает выводы работы Т.С. Всеволодовой-Перель и Т.В. Малининой. Причиной такой сложной картины, вероятно, служит пластичность способов репродукции, которые включают в себя разнообразные виды полового и бесполого размножения; причем даже формы с высокой плоидностью могут размножаться амфимиктически.
Ключевые слова: генетические линии; кариотипы; филогения; дивергенция видов.

Introduction

Polyploidy in animals is relatively rare (Muller, 1925; Orr, 1990). However, certain groups are exceptions to this rule and exhibit a significant incidence of polyploidy (Gregory, Mable, 2005). Earthworms are among these exceptions (Muldal, 1952; Viktorov, 1997): the initial studies demonstrated that polyploidy is observed not only among groups of closely related species, but even within a single species, and often in sympatry (Omodeo, 1952, 1955). Subsequently, this phenomenon was documented in representatives of diverse genera (Vsevolodova-Perel, Bulatova, 2008; Mezhzherin et al., 2018). In addition to that, earthworms demonstrate diverse ways of reproduction (Pavlíček et al., 2023). Although in animals polyploidy is generally associated with parthenogenesis, polyploid earthworms often retain the ability for amphimixis (Viktorov, 1989). While some species comprise a set of races with different ploidy levels, the prevailing view is that this alone is not a sufficient reason to classify them as distinct species (Vsevolodova-Perel, Bulatova, 2008).

Molecular studies revealed a vast genetic diversity within earthworm species (King et al., 2008; Porco et al., 2013). In most cases, several well-defined clades within a given species were identified, with 15–20 % of nucleotide substitutions between mitochondrial genes (these estimates sometimes vary, because different studies employ various distance measures, like Kimura-2-parameter, etc.). These clades are commonly referred to either as cryptic species or as the so-called genetic lineages (Marchán et al., 2018). The attempts to clarify the issue of genetic divergence on the nuclear level using various molecular methods generally confirmed the existence of significant nucleotide distances between these lineages (Martinsson, Erséus, 2017; Taheri et al., 2018), although in some instances, the data did not demonstrate signs of reproductive isolation of distinct lineages differing on the mitochondrial level (Giska et al., 2015; Martinsson et al., 2017; Martinsson, Erséus, 2018).

Thus, we can see that certain earthworm species have multiple races with different ploidy levels, as well as several genetic lineages with distinct mitochondrial and nuclear genomes. However, the relationship between chromosomal and DNA sequence variation remains unclear. Does each chromosomal race correspond to a particular genetic lineage, or do the boundaries between these entities lie elsewhere?

In this review, we analyzed the patterns of chromosomal and molecular variation in several earthworm species from various genera within the family Lumbricidae. The results provide insight into the relationships between these entities and outline directions for future research.

Materials and methods

The data on the chromosome numbers of the populations of various earthworm species were taken from published materials (Muldal, 1952; Omodeo, 1952, 1955; Vedovini, 1973; Graphodatsky et al., 1982; Bulatova et al., 1984, 1987; Perel, Graphodatsky, 1984; Casellato, 1987; Viktorov, 1989, 1997; Kashmenskaya, Polyakov, 2008; Vsevolodova-Perel, Bulatova, 2008; Vlasenko et al., 2011; Mezhzherin et al., 2018). The information on the number of genetic lineages and on the assignment of particular populations to genetic lineages was extracted from scientific papers (Heethoff et al., 2004; King et al., 2008; Porco et al., 2013; Fernández et al., 2016; Shekhovtsov et al., 2014, 2020a–d; Ermolov et al., 2023), as well as the GenBank database.

For *Dendrobaena octaedra* (Savigny, 1826), we also obtained a sequence dataset for 99 specimens from 24 populations from Russia and adjacent countries (Fig. 1). Briefly, earthworms were fixed in ethanol; DNA was extracted from whole individuals or from parts of the body (ca. 100 mg) using BioSilica columns (Dia-m, Russia) according to the manufacturers' instructions. Fragments of the *cox1* gene were amplified using universal primers and sequenced as described in (Shekhovtsov et al., 2013). Sequences were deposited in GenBank under accession numbers OR366494–OR366522, KJ772497, KJ772504, KX400644, MH755642, MH755644, MH755645, MH755647, MH755649, MH755654, MH755666, MH755670, MH755672. A dataset of 157 full-length 658 bp *cox1* barcodes was taken from GenBank. Unique haplotypes were extracted from these datasets. Sequences of *D. octaedra* L2 were additionally searched in the BOLD database (https://v4.boldsystems.org/). Maximum likelihood trees were constructed using RAxML v. v. 8.2.12 (Stamatakis, 2014) with the GTRCAT substitution model and 1000 bootstrap replicates. Bayesian analysis was performed in MrBayes v. 3.4 (Ronquist et al., 2012). Two simultaneous independent runs were performed with 10 million generations each; 25 % of the generations were discarded as burn-in.

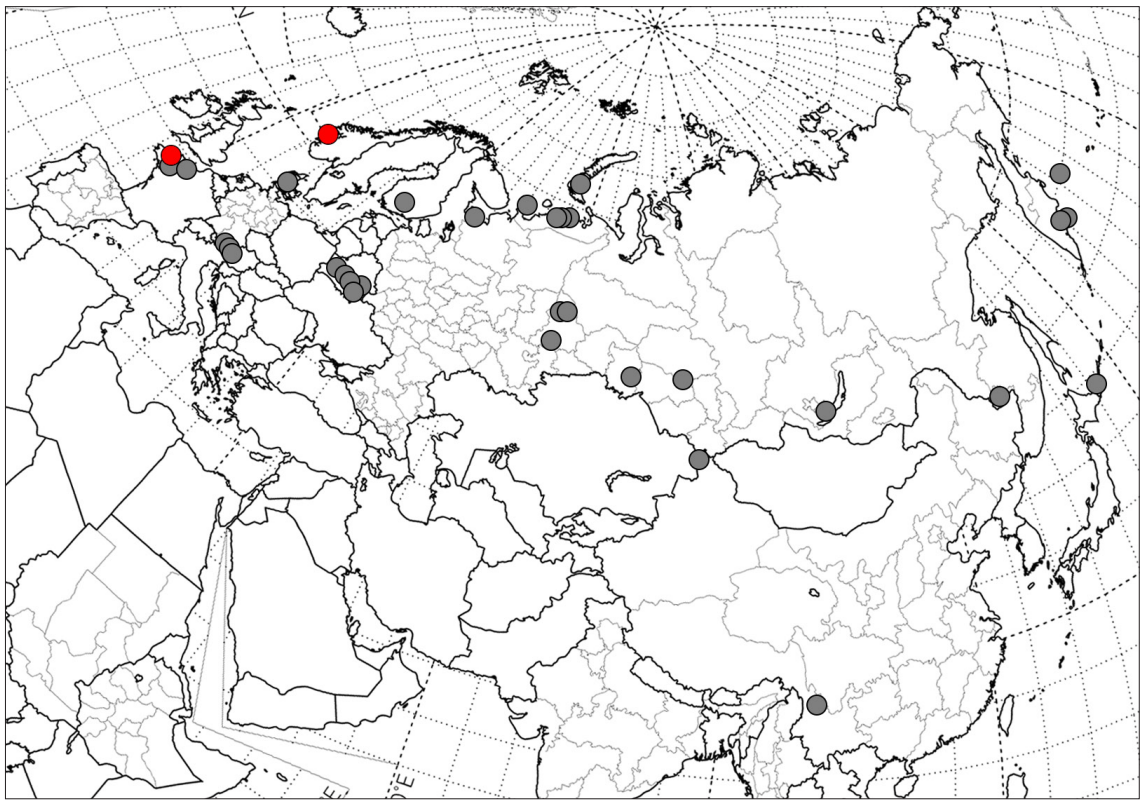

Fig. 1. Sampling locations of the sequenced *D. octaedra* individuals from Eurasia. Russia, Belarus, and Kazakhstan, our data; other countries, GenBank.

Grey dots – lineage 1; red dots – lineage 2.

Results

Dendrobaena octaedra

Among the 99 *D. octaedra* sequences obtained by us, we found 40 unique haplotypes. We also extracted 157 sequences from GenBank with 41 unique haplotypes. We combined these unique haplotypes from the two samples to construct phylogenetic trees (Fig. 2). Our analysis revealed that average genetic diversity within *D. octaedra* is very low compared to other earthworms. The majority of haplotypes belonged to a single group with lower diversity: average p-distance within the group was 2.3 %.

However, two haplotypes from GenBank, MF121744 and MF121754, differed significantly from the rest of the sample with an average p-distance of 19 %. These specimens were designated by the authors as *Dendrobaena octaedra* complex sp. L2. They were collected in the Eawy forest in Normandy (France), near the English Channel. Another three closely related sequences were found in the BOLD database, one from the vicinities of Florelandet (Norway), and the collection points of the other two specimens were undisclosed. These regions were affected by the most recent glaciation, so the local populations of *D. octaedra* were obviously introduced from another region relatively recently (ca. 10 kya). There are too few data on these specimens; since there are no associated papers with morphological descriptions, there is a chance that they might belong to another yet unknown species.

Four chromosomal races are known within *D. octaedra*, $4n = 72$, $5n = 90$, $6n = 108$, and $8n = 144$ (Casellato, 1987; Viktorov, 1993; Mezhzherin et al., 2018). Since the predominant majority of *D. octaedra* populations belong to a single genetic lineage, we can suggest that these three chromosomal races coexist within this lineage.

Aporrectodea rosea (Savigny, 1826)

Races with $2n = 36$, $3n = 54$, $4n = 72$, $5n = 90$, $6n = 108$, $8n = 144$, and $10n = \sim180$ were described for *A. rosea* (Muldal, 1952; Casellato, Rodighiero, 1972; Casellato, 1987; Vsevolodova-Perel, Bulatova, 2008; Vlasenko et al., 2011). The initial barcoding studied uncovered the existence of several genetic lineages within this species: R.A. King et al. (2008) detected three lineages, whereas D. Porco et al. (2013) discovered four. R. Fernández et al. (2016) performed a detailed phylogeographic analysis of *A. rosea* in Western Europe, demonstrating that it can be divided into two major clades: the Eurosiberian and the Mediterranean. The former has a cosmopolitan distribution and includes the four lineages identified by R.A. King et al. (2008) and D. Porco et al. (2013), while the latter is confined to the Mediterranean region. Subsequently, additional genetic lineages were found in Russia and adjacent countries, all belonging to the Eurosiberian clade (Shekhovtsov et al., 2020a).

Therefore, many genetic lineages and ploidy races coexist within *A. rosea*. Detailed data on the relationships between them are currently not available. However, there is a single example that can shed light on this issue: S.V. Mezhzherin et al. (2018) reported a case of four chromosomal races ($2n$, $3n$, $6n$, and $8n$) in the A.V. Fomin Botanical Garden (Kyiv).

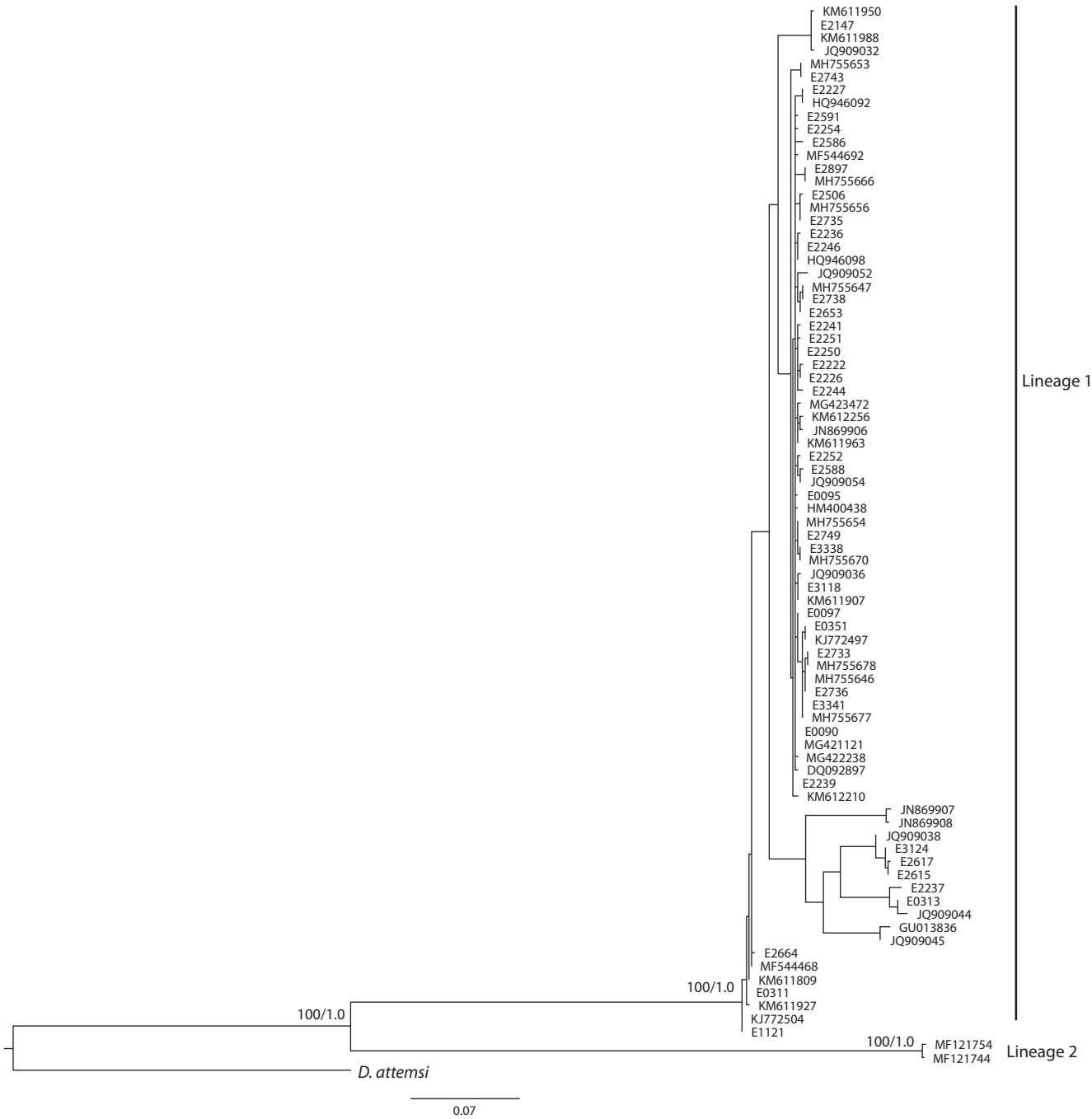

Fig. 2. The phylogenetic tree built for the *D. octaedra* haplotypes using the maximum likelihood method.
Numbers near branches indicate bootstrap support/Bayesian posterior probabilities.

According to phylogeographic studies (King et al., 2008; Shekhovtsov et al., 2020a), three lineages are rarely found in sympatry, and four have never been reported. Therefore, it is plausible that in this case, several chromosomal races coexist within a single lineage. Sure, this cannot be called hard evidence, but we don't have better data so far.

It is worth noting that body size does not correlate with chromosome number in *A. rosea* (Vlasenko et al., 2011). It is presumed that the races with $2n = 36$, as well as at least some populations with $4n = 72$ and $6n = 108$ are amphimictic (Vsevolodova-Perel, Bulatova, 2008).

Bimastos rubidus (Eisen, 1874)

B. rubidus (formerly known as *Dendrodrilus rubidus*) is a rare species containing only a single genetic lineage (Ermolov et al., 2023) despite the fact that it has considerable intraspecific diversity and was until recently considered to contain four subspecies (Holmstrup, Simonsen, 1996; Vsevolodova-Perel, 1997; Sims, Gerard, 1999; Csuzdi et al., 2017). Chromosomal studies reported the presence of six ploidy races within the species: $2n = 34$, $3n = 51$, $4n = 68$, $5n = 85$, $6n = 102$, and $8n = 136$ (Muldal, 1952; Omodeo, 1952; Vedovini, 1973; Casellato, 1987; Mezhzherin et al., 2018). Thus, similar to

D. octaedra, multiple ploidy races are encompassed within a single genetic lineage.

Octolasion tyrtaeum (*lacteum*) (Örley, 1881)

O. tyrtaeum is generally believed to comprise two discrete size groups, referred to as "small" (body length 4–8 cm) and "big" (10–14 cm) (Meinhardt, 1974; Heethoff et al., 2004). Molecular studies demonstrated that mitochondrial and nuclear gene sequences of these two groups are significantly different and belong to two distinct genetic lineages (Heethoff et al., 2004; Shekhovtsov et al., 2014). These two lineages were reported to differ in ploidy: the "small" one is diploid, while the "big" one is triploid (Mezhzherin et al., 2018). Thus, in this case we can observe that a ploidy race corresponds to a single lineage. *O. tyrtaeum* is also a rare example of the dependence of body size on ploidy in earthworms (Mezhzherin et al., 2018).

It should be noted that this division of *O. tyrtaeum* into two groups is not straightforward. A third genetic lineage with body size similar to the "small" lineage but with different body proportions was found (Shekhovtsov et al., 2014, 2020b). Its ploidy is unknown. Moreover, body size may also differ between populations of different lineages (Shekhovtsov et al., 2020b).

Dendrobaena schmidti (Michaelsen, 1907)

D. schmidti is widespread in the Caucasus and adjacent regions. It exhibits a wide range of pigmentation intensity, from unpigmented to deep purple coloration, and body size, ranging from 35 to 160 mm. Due to this variation, many subspecies were isolated from *D. schmidti*, some of them later recognized as distinct species (Perel, 1966; Kvavadze, 1985; Vsevolodova-Perel, 2003). However, not all these subspecies were widely accepted by researchers due to the lack of clear boundaries between them (Vsevolodova-Perel, 2003).

Chromosomal studies demonstrated that all the subspecies of *D. schmidti* distinguished in the book of E.S. Kvavadze (1985) exhibit the same chromosome number of $2n = 36$ (Bakhtadze et al., 2003, 2005). On the other hand, *D. tellermanica*, originally described as *D. s. tellermanica* in 1966 (Perel, 1966) and subsequently elevated to the species rank (Vsevolodova-Perel, 2003), is tetraploid ($4n = 72$) (Bakhtadze et al., 2003, 2005). *D. tellermanica* was distinguished from *D. schmidti* based on the lack of pigmentation, the start of the clitellum on the 25th segment (vs. 26th in *D. schmidti*), and wider distribution beyond the Caucasus region. Initially, it was believed to be strictly parthenogenetic, but later studies revealed the presence of populations with mature spermatozoa and spermatophores (Vsevolodova-Perel, 2003).

Recent molecular studies (Shekhovtsov et al., 2020c, 2023) showed that while *D. schmidti* and *D. tellermanica* are related, they exhibit significant differences in terms of nucleotide substitutions. This implies relatively ancient polyploidization, similar to *O. tyrtaeum*.

Eisenia nordenskioldi (Eisen, 1879) complex

E. nordenskioldi has a vast distribution in Northern Asia and adjacent areas and is known for its high morphological diversity (Malevich, 1956; Vsevolodova-Perel, 1997). Thus it is not surprising that it was found to have extensive genetic diversity (Blakemore, 2013; Shekhovtsov et al., 2013, 2016, 2018; Hong, Csuzdi, 2016). Molecular studies revealed that *E. nordenskioldi* consists of multiple genetic lineages divided into two large clades (Shekhovtsov et al., 2020d). These lineages strongly differ in mitochondrial and nuclear genome sequences (Shekhovtsov et al., 2020c), as well as genome size (Shekhovtsov et al., 2021). Therefore, *E. nordenskioldi* should be regarded as a species complex. Preliminary, this complex was divided into two large clades, referred to as *E. nordenskioldi* s. str. (genetic lineages 6, 7, and 9) and *Eisenia* sp. 1 aff. *E. nordenskioldi* (all other lineages) (Shekhovtsov et al., 2020d).

E. nordenskioldi is probably the best studied model of karyotype diversity among earthworms (Graphodatsky et al., 1982; Bulatova et al., 1984, 1987; Perel, Graphodatsky, 1984; Viktorov, 1989, 1997; Kashmenskaya, Polyakov, 2008; Vsevolodova-Perel, Bulatova, 2008). Races with $2n = 36$, $4n = 72$, $6n = 96$–102, and $8n = 142$–152 were identified (Viktorov, 1997). However, it is not yet clear how the division into genetic lineages correlates with the different ploidy races. Although no direct studies are available, published data allow one to attribute certain populations of *E. nordenskioldi* to specific lineages and races. For example, the population from Magadan with $8n = 152$ chromosomes (Viktorov, 1989) belongs to lineage 9: individuals collected from the same locations were sent to A.G. Viktorov and the authors of this paper by D.I. Berman. Furthermore, extensive studies failed to find other lineages of the pigmented form of *E. nordenskioldi* in this region (Shekhovtsov et al., 2020d).

M.N. Kashmenskaya and A.V. Polyakov (2008) conducted a study on the chromosome set of two individuals identified as *E. n. nordenskioldi* and *E. atlavyniteae*, a closely related species isolated from *E. nordenskioldi* (Perel, Graphodatsky, 1984), from the Central Siberian Botanical Garden in Novosibirsk. Both individuals were found to be diploid ($2n = 36$). A later study of earthworms from the same location found lineages 1, 2, and 3 of the pigmented form of *E. nordenskioldi* (Shekhovtsov et al., 2013). Although we cannot attribute the specimens from the study of M.N. Kashmenskaya and A.V. Polyakov (2008) to a precise lineage, we know that this location does not harbor any lineage of *E. nordenskioldi* s. str. Therefore, these diploid populations belong to *Eisenia* sp. 1 aff. *E. nordenskioldi*.

Tetra- and octoploid races of *E. nordenskioldi* were reported from the Taymyr Autonomous Okrug, located in the north of West Siberia (Bulatova et al., 1984; Viktorov, 1989; Vsevolodova-Perel, Leirikh, 2014). Molecular studies identified genetic lineages 1 and 9 of the pigmented form of *E. nordenskioldi* from the same region (Shekhovtsov et al., 2020d). T.V. Malinina and T.S. Perel (1984) used allozyme data to demonstrate that the octoploid population from Taymyr is related to those from the south of West Siberia compared to other regions, suggesting that it likely belongs to lineage 1.

The population of *E. nordenskioldi* from the Dzhanybek experimental station of the Institute of Forest Science RAS, located in the steppe zone of European Russia in Volgograd Oblast, has been reported to have $4n = 72$ (Malinina, Perel, 1984; Viktorov, 1989). This population was artificially introduced from the floodplain of the Eruslan River in Saratov Oblast, Russia (Vsevolodova-Perel, Bulatova, 2008). According to our data, only lineage 7 of *E. nordenskioldi* is found in

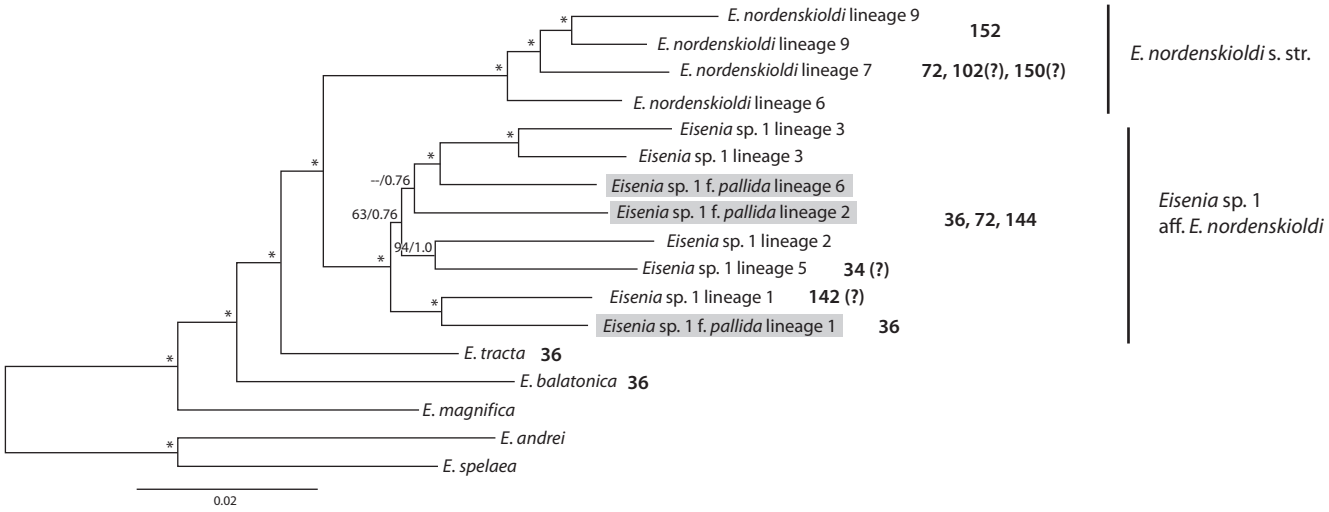

Fig. 3. The position of chromosomal races of *E. nordenskioldi* on the phylogenetic tree of the species according to published data. The tree was taken from (Shekhovtsov et al., 2020d) built using transcriptomic data of various genetic lineages of *E. nordenskioldi* and outgroup species using the maximum likelihood algorithm.

Grey boxes around lineage names indicate that these lineages belong to the *pallida* (unpigmented) form. Bold numbers indicate chromosome numbers; question signs indicate that karyotype assignment is tentative. Numbers near branches indicate bootstrap support/Bayesian posterior probabilities. * Refers to 100/1.0.

this region. The population from the Prioksko-Terrasny Nature Reserve in Moscow Oblast is also within the distribution range of lineage 7. Moreover, T.V. Malinina and T.S. Perel (1984) suggested that it is related to the Dzhanybek population based on allozyme data, so we could also attribute it to lineage 7. The same can be hypothesized for the Kursk population, which has $6n = 102$ chromosomes (Viktorov, 1989).

A.G. Viktorov (1989) reported that *E. nana* from East Kazakhstan Oblast has 34 chromosomes. It has since been discovered that this species is actually a synonym of lineage 5 of the pigmented form of *E. nordenskioldi* (Shekhovtsov et al., 2020d; Golovanova et al., 2021). As the individuals used for both genetic and chromosomal analyses were collected from the same region, it is reasonable to hypothesize that they belong to the same lineage.

The unpigmented *pallida* form of *E. nordenskioldi* is considered to be diploid (Vsevolodova-Perel, Leirikh, 2014). These data were obtained for the population from the Novosibirsk Akademgorodok (Malinina, Perel, 1984; Viktorov, 1989). However, the *pallida* form is distributed throughout Siberia and the Far East and also contains many genetic lineages with different distributions (Shekhovtsov et al., 2016). The *pallida* form from Akademgorodok belongs to lineage 6 (Shekhovtsov et al., 2020d). It has a small genome size (ca. 270 Mb) (Shekhovtsov et al., 2021), while lineage 1 of the *pallida* form has a big genome (ca. 2500 Mb), suggesting that it may be polyploid.

We summarized the obtained data in Fig. 3, which includes chromosome numbers and the phylogenetic tree constructed using 212 nuclear genes (Shekhovtsov et al., 2020d). However, it is important to note that the chromosome numbers displayed are representative of certain populations and may not apply to the entire lineage.

Eisenia tracta, the sister species of the *E. nordenskioldi* complex, has $2n = 36$ chromosomes, as does its relative *E. balatonica* (Fig. 3). Therefore, it is reasonable to suggest that the ancestors of the two clades of *E. nordenskioldi* were also diploid with 36 chromosomes, and that polyploidy arose independently in both clades. This hypothesis on the independent origin of polyploid races in *E. nordenskioldi* was proposed by T.V. Malinina and T.S. Perel (1984) based on allozyme data. The authors concluded that octoploid populations arose independently at least twice. Our data supports this position, as octoploid races appear to have arisen independently in the two large clades of *E. nordenskioldi* (Fig. 3).

It is worth noting that published papers (Viktorov, 1997; Vsevolodova-Perel, Bulatova, 2008) and our unpublished data indicate that all studied populations of *E. nordenskioldi* have well-developed testes and normal spermatogenesis. According to A.G. Viktorov (1997), the evidence for parthenogenesis was only observed for the *acystis* form from Central Asia, which was subsequently isolated into a separate species (Vsevolodova-Perel, 1997). Additionally, octoploid individuals of lineage 9 from Magadan were observed copulating (D.I. Berman, personal communication). Thus, the available evidence suggests that polyploidy does not result in the loss of sexual reproduction in the *E. nordenskioldi* complex in most cases.

Discussion

Based on the data presented above, it is apparent that the relationships between ploidy races and genetic lineages are rarely straightforward. This was only observed in the cases of *O. tyrtaeum* and the *D. schmidti – D. tellermanica* pair. For many species, multiple ploidy races were found to belong to the same genetic lineage. In all these cases, races of different ploidy do not have any apparent differences on the level of mitochondrial or nuclear DNA, suggesting that polyploidization events in these cases may be recent. However, in other cases, such as the *E. nordenskioldi* complex, the age of the polyploidization events is unknown, but is likely to be significant, 1–3 Mya as estimated in (Shekhovtsov et al., 2013).

Although the precision of molecular clock dating using only mitochondrial data and no fossils is limited (Kodandaramaiah, 2011), deep divergence between these taxa is obvious.

Earthworms exhibit a high degree of plasticity in their modes of reproduction: most species are reported to have either amphimixis or parthenogenesis, as well as less common modes such as autogamy or restitutional automixis (Pavlíček et al., 2023). Although it is generally considered that polyploidy in animals should be associated with parthenogenesis, there is no obvious association between these modes in earthworms: many polyploid races retain the ability to reproduce sexually. Furthermore, populations with sexual reproduction or partial degeneration of the sexual system were found in species that are considered parthenogenetic (Fernández et al., 2010). In other parthenogenetic species, there are genetic clues to possible sexual reproduction (Simonsen, Holmstrup, 2008). This flexibility in reproduction modes may contribute to the widespread occurrence of polyploidy in earthworms.

Some researchers suggested that polyploid races may have arisen as a result of allopolyploidization (Mezhzherin et al., 2018). However, it is important to note that none of the available molecular studies have yet provided evidence to support this hypothesis.

Conclusion

Based on the available data, we can conclude that the most frequent case in earthworms is "one genetic lineage – several ploidy races", implying that this polyploidy is recent. However, in some instances, polyploid populations can survive for prolonged periods of time, giving rise to new genetic lineages.

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Phylogeography of the woolly mammoth (*Mammuthus primigenius*) in the Minusinsk Depression of southern Siberia in the Late Pleistocene

S.A. Modina[1, 2], M.A. Kusliy[1], D.G. Malikov[1, 3], A.S. Molodtseva[1, 4]

[1] Institute of Molecular and Cellular Biology of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
[2] Novosibirsk State University, Novosibirsk, Russia
[3] V.S. Sobolev Institute of Geology and Mineralogy of the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
[4] Institute of Archaeology and Ethnography, Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia
s.modina@g.nsu.ru

Abstract. To date, a number of studies have been published on the phylogenetics of woolly mammoths (*Mammuthus primigenius*), ranging from analyses of parts of the mitochondrial genome to studies of complete nuclear genomes. However, until recently nothing was known about the genetic diversity of woolly mammoths in southern Siberia, in the Minusinsk Depression in particular. Within the framework of this effort, libraries for high-throughput sequencing of seven bone samples of woolly mammoths were obtained, two-round enrichment using biotinylated probes of modern mtDNA of *Elephas maximus* immobilised on magnetic microspheres and sequencing with subsequent bioinformatic analysis were carried out. Phylogenetic reconstructions showed the presence of all studied mammoths in clade I, which expanded its range. The assignment of mammoth mitotypes in the Minusinsk Depression to different clusters within clade I may indicate a sufficiently high diversity of their gene pool. Phylogeographic reconstructions revealed a genetic proximity of mitochondrial lineages of Late Pleistocene mammoths of the Minusinsk Depression and other regions of eastern Siberia and estimated their divergence time in the range of 100–150 thousand years ago, which indicates active migrations of woolly mammoths over vast territories of eastern Siberia in the late Middle Pleistocene-early Late Pleistocene.

Key words: ancient DNA; woolly mammoth; phylogeography; mitochondrial genome; southern Siberia.

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Филогеография шерстистого мамонта (*Mammuthus primigenius*) в Минусинской котловине на Юге Сибири в позднем плейстоцене

С.А. Модина[1, 2], М.А. Куслий[1], Д.Г. Маликов[1, 3], А.С. Молодцева[1, 4]

[1] Институт молекулярной и клеточной биологии Сибирского отделения Российской академии наук, Новосибирск, Россия
[2] Новосибирский национальный исследовательский государственный университет, Новосибирск, Россия
[3] Институт геологии и минералогии им. В.С. Соболева Сибирского отделения Российской академии наук, Новосибирск, Россия
[4] Институт археологии и этнографии Сибирского отделения Российской академии наук, Новосибирск, Россия
s.modina@g.nsu.ru

Аннотация. К настоящему времени опубликован ряд исследований о филогенетике шерстистых мамонтов (*Mammuthus primigenius*), начиная с анализа частей митохондриального генома и заканчивая изучением полных ядерных геномов. Однако до сих пор ничего не известно о генетическом разнообразии шерстистых мамонтов на Юге Сибири, в частности в Минусинской котловине. В рамках данной работы были получены библиотеки для высокопроизводительного секвенирования семи костных образцов шерстистых мамонтов, проведены двухраундное обогащение с использованием биотинилированных зондов современной мтДНК *Elephas maximus*, иммобилизованных на магнитные микросферы, и секвенирование с последующим биоинформационным анализом. Филогенетические реконструкции показали принадлежность всех исследованных нами вариантов мтДНК мамонтов к кладе I, что расширило ее ареал. Расположение митотипов мамонтов Минусинской котловины в разных субкладах внутри клады I может указывать на достаточно высокое разнообразие их генофонда. Филогео-

графические реконструкции выявили генетическую близость митохондриальных линий позднеплейстоценовых мамонтов Минусинской котловины и других регионов Восточной Сибири и их дивергенцию во временном промежутке от 100 до 150 тыс. лет назад, что свидетельствует об активных миграциях шерстистых мамонтов на обширных территориях Восточной Сибири в конце среднего–начале позднего плейстоцена.
Ключевые слова: древняя ДНК; шерстистый мамонт; филогеография; митохондриальный геном; Южная Сибирь.

Introduction

The phylogeography of the woolly mammoth, one of the most important representatives of the mammoth fauna, is currently being studied on an extensive scientific basis. The Genbank database contains 32 mitogenomes of *Mammuthus primigenius* Blumenbach, 1799. According to palaeontological data, the common lineage of Asian elephants and woolly mammoths (*Mammuthus primigenius*) diverged from the lineage of African elephants (*Loxodonta africana*) 6 million years ago, and the divergence of the lineages of mammoths and Asian elephants (*Elephas maximus*) is dated by genetic data to 440 thousand–2 million years ago (Krause et al., 2006; Rogaev et al., 2006).

In 2007, the results of one of the first studies of the phylogeographic relationships of mitochondrial lineages from a large and diverse sample of woolly mammoths were published, based on mixed sequence analysis of 741 bp of the mitogenome (three genes plus part of the control region) (Barnes et al., 2007). The sample included 41 woolly mammoths from Europe, Asia (western Beringia, Kamchatka Peninsula, north-central Siberia) and North America (eastern Beringia). The samples ranged in age from 12 to 51 thousand years. The study identified two major mitogroups of woolly mammoths that existed in western and eastern Siberia, the Far East and Alaska, as well as a mitochondrial lineage of mammoths from the European region. The first mitogroup was distributed in Siberia and North America, while the second was restricted to the north of eastern Siberia, between the Lena and Kolyma river valleys. Sequence analysis of the 743 bp hypervariable region of the mitogenome of 160 mammoths from the Holarctic region of Eurasia and North America revealed 80 haplotypes forming five haplogroups (A–E), which form three major clades (A, B and C+D+E), the clustering of which is supported by high posterior probabilities. Clade A contains only Asian mitotypes, clade C contains only North American mitotypes, and the remaining haplogroups are mixed (Debruyne et al., 2008). However, the studies discussed above only focused on partial mitogenome sequences, which, unlike complete sequences, do not provide such a clear resolution of phylogeny.

Studies of 18 complete woolly mammoth mitogenomes confirmed the presence of two mitogroups in Siberia during the Late Pleistocene (Krause et al., 2006; Poinar et al., 2006; Rogaev et al., 2006; Gilbert et al., 2007). One of the clades was stably represented in the gene pool of the populations for a long time, while representatives of the second clade became extinct. The disappearance of the second clade may be related to its limited distribution (Payne, Finnegan, 2007).

There is disagreement about the timing of intraspecific divergence of mammoths. Some researchers suggest 1–2 million years ago (Gilbert et al., 2007), while others suggest around 1 million years ago based on phylogenetic reconstructions (Van der Valk et al., 2021). The representativeness of data on the diversity of mitochondrial DNA variants belonging to clades I and II is low, especially for Siberian populations; therefore, further study of local series of mtDNA samples from different regions is necessary for a complete understanding of the genetic diversity of woolly mammoths in this region.

Nuclear genome analyses have confirmed the closeness of woolly mammoths to Asian elephants (Greenwood et al., 1999; Capelli et al., 2006; Miller et al., 2008)) and estimated the time of divergence of mammoths and African elephants (*Elephas maximus*) at 5–6 million years ago (Poinar et al., 2006). Separate studies of nuclear genome sequences also suggest two mammoth lineages that diverged 1.5–2 million years ago (Miller et al., 2008). Whole-genome analyses, however, suggest that the split occurred between 50 and 155 thousand years ago (Palkopoulou et al., 2015). Studies of the nuclear genomes of Early and Middle Pleistocene mammoths also suggest the existence of two lineages in eastern Siberia, only one of which represents the ancestor of the woolly mammoth.

It should be emphasized that the samples studied so far from Siberia and the Far East are from the northern and eastern regions. Molecular genetic studies of samples from geographically isolated areas are revealing new genetic diversity, such as the presence of a second mitogroup of woolly mammoths in the northern part of eastern Siberia (Gilbert et al., 2007). Additional analyses of mammoth DNA samples from different regions of Siberia are allowing us to expand our understanding of the phylogenetic diversity of mammoth mtDNA and the specifics of its phylogeography. For example, a woolly mammoth genetic lineage was discovered in Taymyr that was previously thought to be characteristic only of Europe (Maschenko et al., 2017). Mammoths of southern Siberia remain understudied at the molecular genetic level, although these data are important for assessing the peculiarities of local mammoth genetic diversity and the specifics of the evolution of regional mammoth populations. To fill this gap, we studied the ancient DNA of woolly mammoths from the Minusinsk Depression.

Working with ancient DNA is challenging due to its low content, degradation and chemical changes, and possible contamination of samples by microorganisms (Pääbo et al., 2004; Brotherton et al., 2007; Carpenter et al., 2013). One of the key approaches to overcome these difficulties is the enrichment of genomic libraries with targeted DNA fragments.

Hybridisation capture has a number of advantages over PCR (Meyer, Kircher, 2010; Horn, 2012). Hybridisation capture involves the preparation of a genomic library and target DNA fragments, their hybridisation and subsequent separation using magnetic particles. Hybridisation capture methods such as primer extension capture or multiplex capture of target fragments have been shown to be fast and efficient (Briggs et al., 2009; Maricic et al., 2010).

In our study, we use the enrichment method proposed by T. Maricic, M. Whitten and S. Pääbo (Maricic et al., 2010)

with two rounds of hybridisation, which has been shown many times (Reich et al., 2010; Dabney et al., 2013; Thalmann et al., 2013; Vorobieva et al., 2020; Kusliy et al., 2021) to be a highly efficient approach for the analysis of the complete mitochondrial genome in ancient samples.

Materials and methods

The material for the study was collected by D.G. Malikov during expeditionary work in 2011–2021, as well as partially obtained from the collections of the Zoological Museum of the N.F. Katanov Khakass State University (ZM KSU) and the L.R. Kyzlasov Khakass National Museum of Local Lore (KNMLL). Territorially, the bone remains cover all parts of the Minusinsk Depression (Fig. 1) and come from six localities of different geological age (see the Table).

^{14}C dates were obtained for all samples (except MAM3) and were previously published in a summary (Malikov et al., 2023). Dating was performed at the Laboratory of Cenozoic Geology, Palaeoclimatology and Mineralogical Indicators of Climate, V.S. Sobolev Institute of Geology and Mineralogy (IGM) SB RAS by the benzene scintillation method. For specimen MAM3 from the Pervomayskoye locality, the age was determined on the basis of ^{14}C dates obtained from other *M. primigenius* remains from this locality with similar preservation of bone material. For radiocarbon dating and DNA extraction, different parts of the same bone remains were used, which were not pre-treated with chemical reagents.

The isolation of ancient DNA from bone powder was performed according to the protocol described in the article by H. Yang et al. (Yang et al., 1998). Observance of all criteria of

Fig. 1. Map of locations of remains of woolly mammoths (*Mammuthus primigenius*) from the Minusinsk Depression.
Locations (red circles) are marked with numbers that correspond to the numbers in the Table (Malikov et al., 2023).

Information on bone material

Number	Location	Geographical coordinates		Age				Type of bone material	Sample name
		N.S.	E.D.	^{14}C	Lab cipher	Cal BP median	Cal BP 95.4 %		
1	Sargov ulus	54.110567	91.463749	14,220 ± 160	COAH-9890	17,306	17,866–16,920	Scapula	MAM1
2	Izykh	53.569545	91.491608	17,955 ± 280	COAH-9783	21,777	22,395–21,005	Tusk	MAM2
3	Pervomayskoye	54.614021	90.947409	*	*		25,020–21,800	Cranium	MAM3
4	Novoselovo alluvial	55.041728	91.024450	16,710 ± 110	COAH-9549	20,200	20,465–19,910	Pelvis	MAM4
5				20,490 ± 170	COAH-9550	24,659	25,125–24,201	Scapula	MAM5
6	Oya	53.395078	92.073517	27,505 ± 240	COAH-9548	31,465	31,881–31,121	Humerus	MAM6
7	Chernousov log	54.673669	90.757873	16,760 ± 135	COAH-9673	20,256	20,551–19,893	Costa	MAM7

* Data obtained from other samples from the collection.

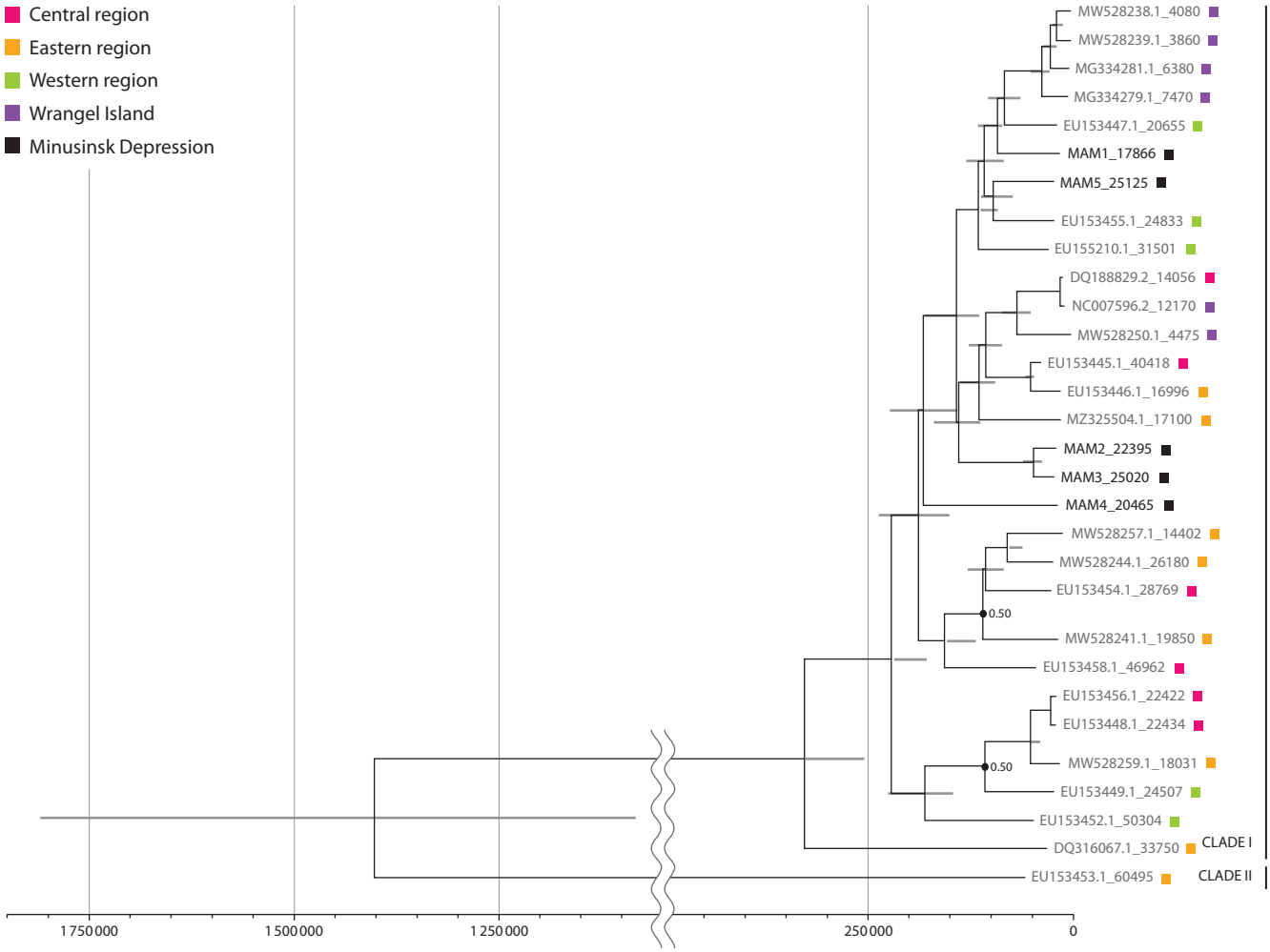

Fig. 2. BEAST phylogenetic reconstructions based on mtDNA sequences of five woolly mammoths from the Minusinsk Depression and 25 previously published mtDNA sequences of woolly mammoths from the Genbank database.

The phylogenetic tree was constructed using the BEAST software platform with internal calibration of branch divergence time based on radiocarbon dating of samples. The colours of the squares reflect the geographical origin of the samples: Central region – part of Siberia washed by the Kolyma and Lena rivers; Eastern region – part of Siberia east of the Kolyma River; Western region – part of Siberia east of 70° E and west of the Lena River; Wrangel Island – a group of islands in northeastern Siberia. The Bayesian posterior probability of the tree topology is greater than 0.75 in all cases except where this is indicated as numbers next to the tree nodes. The light grey lines through the tree nodes denote the standard deviation of the median estimates of divergence times. The radiocarbon dating of each specimen is given next to the name of each specimen after the "_" sign.

purity and authenticity of the DNA samples obtained (Gilbert et al., 2007). As part of this work, we obtained mitogenomic libraries for high-throughput sequencing from seven woolly mammoths from the Minusinsk Depression (southern Siberia), 17–30 thousand years old, using the TruSeq Nano Library Prep Kit (Illumina) according to the manufacturer's protocol. For these libraries, we performed a two-round enrichment by hybridisation with biotinylated fragments of modern mitochondrial DNA from *Elephas maximus* L., 1758, immobilised on Dynabeads® Streptavidin magnetic particles (Life Technologies, USA), which allowed us to significantly increase the proportion of endogenous ancient mitochondrial DNA.

Results

The characteristics of seven sequences of mitogenomes of Late Pleistocene woolly mammoths from the Minusinsk Depression studied by us are presented in the summary table (https://docs.google.com/spreadsheets/d/1XaSB-cb14rxNy0aas5xDLUiI_YiKeBSy-_Kwe1Rt2KQ/edit?usp=sharing). The average depth of coverage of the characterised mitogenomes varies from 0.5 to 15.5x, and the width of coverage ranges from 38 to 99.5 % of the reference mitogenome length. The average percentage of uniquely mapped pooled reads to total pooled reads is 7.9 %. Based on the values of base deamination frequency and average size of DNA fragments obtained, we can conclude that the mammoth bone samples from the Minusinsk Depression have a high degree of DNA preservation, most likely due to relatively good environmental conditions for DNA preservation.

Only specimens with sufficient breadth (more than 70 %), depth of mitogenome coverage (more than 2) and radiocarbon dates were used to construct a phylogenetic tree with a certain time of divergence of genetic lineages (Fig. 2). These criteria were met by five of the seven specimens examined. The same selection criteria were used to include sequences

from previously published woolly mammoth mitogenomes in the analysis. The analysis was performed using the BEAST software platform, based on the topology of a constructed tree with an uncertain time of divergence of genetic lineages.

The divergence of the genetic lineages of woolly mammoths from the Minusinsk Depression from the most genetically similar mammoths from other regions of eastern Siberia occurred in the time interval between 150 and 100 thousand years ago. Woolly mammoths from the Minusinsk Depression form sister clades with woolly mammoths from other represented regions of Siberia (Wrangel Island, central, western and eastern regions), which distinguishes them from some other studied local groups of mammoths, such as the mammoths from Wrangel Island, which were in a stage of reduced genetic diversity.

Discussion

This study allows us to estimate the mitochondrial genetic diversity of woolly mammoths in the Minusinsk Depression. The phylogenetic reconstruction obtained shows that the divergence of two clades of woolly mammoths occurred 1–2 million years ago, which correlates with the results of studies of complete mammoth mitochondrial and nuclear genomes described in the introduction (Gilbert et al., 2008; Miller et al., 2008). The divergence of the genetic lineages of mammoths from the Minusinsk Depression and the most genetically similar genetic lineages of mammoths from other regions of eastern Siberia occurred in the time interval from 150 to 100 thousand years ago. The structure of the phylogenetic tree we constructed indicates that the mtDNA sequences of woolly mammoths from the Minusinsk Depression do not form a separate clade on the tree, but are dispersed in different clusters of clade I. At the same time, mtDNA sequences of mammoths from the Minusinsk Depression form sister clades with mtDNA sequences of woolly mammoths from other represented regions of Siberia (Wrangel Island, central, western and eastern regions), which may indicate intensive mammoth migrations across large areas of eastern Siberia in the late Middle to early Late Pleistocene.

The placement of mitotypes of Late Pleistocene mammoths from the Minusinsk Depression in different clades within clade I, in contrast to Holocene mammoths from Wrangel Island, indicates a low probability that they were on the verge of extinction during this period. At this stage, we propose two possible explanations for their position on the phylogenetic tree: 1) the samples studied belong to a single (permanent in the region) population of mammoths characterised by high phylogenetic diversity of mtDNA; 2) the samples studied were obtained from representatives of different mammoth populations that migrated independently through the Minusinsk Depression during the Late Pleistocene. At this stage we have arguments "for" and "against" each of the versions. For example, the fact that some of the specimens from localities of different geological age form either single or closely related clades (Fig. 2), regardless of their geological age and location, may support the idea that the mammoths of the Minusinsk Depression studied by us belong to a single population. In addition, the revealed isotopic signal of carbon and nitrogen stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) in mammoths from the Minusinsk Depression differs significantly from those in northern populations of the species (Malikov et al., 2023). This suggests that the animals lived in this region for a relatively long time, which is reflected in their isotopic indices.

At the same time, the wide dispersal of the mammoths studied on the general phylogenetic tree may indicate that they belonged to different populations. In support of this version, it should be noted that there are currently no mammoth finds in the region under consideration that can be confidently attributed to warm Late Pleistocene. It is possible that during the warm intervals of the Late Pleistocene, conditions in southern Siberia were unfavourable for the permanent habitat of *M. primigenius*. In this case, representatives of the species could only repeatedly migrate into the depression during cold periods. Furthermore, modern African elephants are known to live in small groups of 6–8 individuals with seasonal home ranges of 130–1,600 km^2 (Nasimovich, 1975). However, under unfavourable conditions, individual elephant movements can reach 32,000 km^2 per year (Wall et al., 2013). The total area of the Minusinsk intermountain trough (including the Nazarovskaya Depression) is approximately 100,000 km^2 (Vorontsov, 2012). The maximum length of the Minusinsk Depression in the northwestern direction is about 450 km, with a maximum width (along the southern Minusinsk trough) of ~400 km. Consequently, the total area of the region is only sufficient to support a small population of large animals such as mammoths. This suggests that the area of the Minusinsk Depression is insufficient to support permanent populations of *M. primigenius*. This is because the resource base of the depression is limited and the annual seasonal migrations of the species are comparable to or exceed the size of the depression itself.

Another argument for the migratory nature of the Minusinsk Depression mammoth population is the fact that two samples from the Novoselovo alluvial site (MAM4 and MAM5) showed maximum genetic distance (Fig. 2). On the contrary, samples from the Pervomayskoye (MAM3) and Izykh (MAM2) localities formed a single group. Although the sites are more than 100 km apart, they date to approximately the same time interval (about 21.8 thousand years ago). It is possible that these individuals belong to a single population that migrated into the region from time to time, possibly over a long period of time.

If the second concept is true, the data obtained can be regarded as confirmation of the local extinction of mammoths in the Minusinsk Depression at the Pleistocene-Holocene boundary, which was probably caused by the development of taiga and forest-steppe landscapes in western and eastern Siberia. As a result, the replenishment of populations of herbivorous mammals of the Minusinsk Depression and their seasonal migrations stopped (Malikov, 2015).

Conclusion

In summary, climate change from the Late Glacial to the Holocene resulted in a reduction of open areas in Eurasia, which in turn reduced the habitat area of mammoths and other steppe animals. This process involved complex changes in climate and vegetation in space and time, the survival of species in refugia, local extinctions and temporary expansion of habitats.

One of the most effective approaches to a detailed reconstruction of these processes is the study of local series of

mammoth mitochondrial DNA samples belonging to different chronological periods. Our study is a step in this direction.

It is necessary to continue palaeontological and molecular genetic studies of woolly mammoths in isolated regions of Siberia in order to fully determine their genetic diversity and the causes of their extinction in this locality. It is preferable to study complete genomes, which will make phylogeographic analyses more accurate and reliable.

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Сибирского отделения Российской академии наук»,
проспект Академика Лаврентьева, 10, Новосибирск, 630090.
Адрес редакции: проспект Академика Лаврентьева, 10, Новосибирск, 630090.
Секретарь по организационным вопросам С.В. Зубова. Тел.: (383)3634977.
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