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Here and there: the double-side transgene localization

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Abstract. Random transgene integration is a powerful tool for developing new genome-wide screening approaches. These techniques have already been used for functional gene annotation by transposon-insertion sequencing, for identification of transcription factor binding sites and regulatory sequences, and for dissecting chromatin position effects. Precise localization of transgenes and accurate artifact filtration are essential for this type of method. To date, many mapping assays have been developed, including Inverse-PCR, TLA, LAM-PCR, and splinkerette PCR. However, none of them is able to ensure localization of both transgene's flanking regions simultaneously, which would be necessary for some applications. Here we proposed a cheap and simple NGS-based approach that overcomes this limitation. The developed assay requires using intentionally designed vectors that lack recognition sites of one or a set of restriction enzymes used for DNA fragmentation. By looping and sequencing these DNA fragments, we obtain special data that allows us to link the two flanking regions of the transposon. This can be useful for precise insertion mapping and for screening approaches in the field of chromosome engineering, where chromosomal recombination events between transgenes occur in a cell population. To demonstrate the method's feasibility, we applied it for mapping SB transposon integration in the human HAP1 cell line. Our technique allowed us to efficiently localize genomic transposon integrations, which was confirmed via PCR analysis. For practical application of this approach, we proposed a set of recommendations and a normalization strategy. The developed method can be used for multiplex transgene localization and detection of rearrangements between them.
Key words: transgenesis; genome-wide screening; transgene mapping; sleeping beauty transposon.

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Здесь и там: двусторонняя локализация интеграций трансгена

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Аннотация. Полногеномные скрининговые методы, основанные на случайной интеграции экзогенных генетических конструкций, – новейший класс инструментов, открывающий возможности для изучения широкого спектра геномных процессов. Данный подход уже был применен к функциональному аннотированию генов млекопитающих, скринингу приспособленности бактерий, определению сайтов связывания факторов транскрипции, идентификации регуляторных генетических элементов и исследованию хромосомного эффекта положения. Все эти эксперименты требуют точной локализации трансгенов в геноме. Существующие на сегодняшний день методы картирования, такие как Inverse-PCR, TLA, splinkerette PCR и LAM-PCR, не позволяют одновременно определять оба участка генома, фланкирующие одну интеграцию трансгена, что ограничивает применимость подходов, в том числе связанных с хромосомной инженерией. В настоящей работе мы предлагаем метод, с помощью которого можно преодолеть это ограничение. Разработанная технология основана на фрагментации геномной ДНК, не затрагивающей интеграции трансгена. Это достигается путем исключения из последовательности вектора сайтов узнавания одного или нескольких ферментов рестрикции. Затем, как и в Inverse-PCR, были закольцованы молекулы лигированием в разбавленной смеси и секвенированы. Полученные данные дают возможность с высокой точностью идентифицировать перестройки и отделить их от артефактов лигирования, и, кроме того, отследить события транслокаций между интеграциями трансгенов. Это может быть использовано в экспериментах по изучению индуцируемых хромосомных перестроек. Для доказательства применимости метода мы с его помощью картировали интеграции транспозона Sleeping Beauty в клетки человека линии Hap1. Картированные интеграции были валидированы с помощью ПЦР-анализа. В статье приведен ряд рекомендаций для практического использования этого метода в экспериментах по множественной локализации интеграций трансгенных конструкций.
Ключевые слова: трансгенез; полногеномный скрининг; локализация трансгена; транспозон «Спящая красавица».

Introduction

Genome-wide screening assays are important tools for modern genetics and genomics. Many of these methods rely on the integration of exogenous sequences in unknown or random genomic regions, mostly via retroviral or transposon vectors. This approach has already been used for functional gene annotation by transposon-insertion sequencing (Deutschbauer et al., 2011; Goodman et al., 2011; Goh et al., 2017; Cain et al., 2020), for transcription factor binding sites (Wang et al., 2012; Moudgil et al., 2020) and regulatory sequences identification (Pindyurin et al., 2015), and for chromatin position effects dissection (Akhtar et al., 2013).

For all of these techniques, accurate localization of transgene integration sites is crucial. There are several well-established methods for massive parallel genomic mapping of integration sites, from Nanopore (Li et al., 2019; Nicholls et al., 2019) or whole-genome Next Generation Sequencing (NGS) (Zhang et al., 2012; Zastrow-Hayes et al., 2015; Park et al., 2017) to cheaper target PCR-mediated approaches, including Inverse-PCR (Akhtar et al., 2013), LAM-PCR (Gabriel et al., 2014; Wang et al., 2016), splinkerette PCR (Friedrich et al., 2017), and TLA (de Vree et al., 2014; Laboulaye et al., 2018).

Importantly, current NGS-based methods cannot capture both transgene-flanking regions (5′ and 3′) simultaneously. Double-side localization is useful for artifact filtration and detection of translocation events occurring during the integration process, which could confound certain experiments (Francke et al., 1992). Furthermore, this is useful for screening approaches in the field of chromosome engineering, where chromosomal recombination events between transgenes occur in a cell population, such as Scramble technique (Dymond, Boeke, 2012; Hochrein et al., 2018) and others (Smith et al., 1995; Uemura et al., 2010). Conventional Inverse-PCR, routinely employed for transgene insertion identification, is unable to differentiate cases of normal insertion and exchange of flanking regions between different integrations in multiplex analysis. Despite its rarity in the standard conditions, a number of developing methods requires a precise detection of these events. Our approach provides double-sided transgene localization that can be applied for translocation detection between transgene integration points.

Here we developed a cheap Inverse-PCR-based approach enabling us to link 5′ and 3′ transposon flanking regions for all integration sites simultaneously. To demonstrate the method's feasibility, we applied it for mapping SB transposon integration in the human HAP1 cell line. Our technique allowed us to efficiently localize genomic transposon integrations. For practical application of this approach, we suggested a set of recommendations and a normalization strategy. The developed method can be used for multiplex transgene localization and detection of rearrangements between them.

Materials and methods

Plasmid vectors. The Sleeping Beauty transposon vector pSB_LoxP was generated via Gibson Assembly (NEB) by amplifying ITR sites from pSBbi-GP (Addgene #60511) and LoxP-rtTA sequence from pLeGO-rtTA (kind gift from Dr. A.M. Yunusova) and integrating into the pJET 1.2 vector (ThermoFisher, USA). We used PCR-mediated mutagenesis to substitute C to G in the CATG sequence within the right ITR (*Fae*I site). This resulted in the vector used for genomic transposon integration.

A vector expressing SB100X transposase was from Addgene (#34879). To allow selection of transposase-expressing cells, IRES-GFP cassette was amplified from vector Cre-IRES-PuroR (Addgene #30205) and inserted between transposase coding sequence and polyA signal using NEB Gibson Assembly, resulting in pSB100X-GFP vector.

Cell culture and Neon transfection. HAP1 cells were cultured in IMDM with 10 % FBS and 1xPen/Strep (Gibco, USA) according to manufacturer recommendations. Fluorescence-activated cell sorting (FACS) and subcloning was performed on BD FACSAria™ III sorter on 96-well plates or manually. Transfections were done on the Neon transfection system under the following conditions: 1400 V, 20 ms 1 pulse and 1.5 µg total plasmid DNA (transposase expression and transposon carrying plasmid ratio 1:4). On day 2 after transfection, we performed a cell sorting of GFP positive cells.

PCR. For PCRs, qPCR and Double-side Inverse-PCR genomic DNA was extracted using a standard phenol-chloroform extraction protocol. All PCR procedures were performed using the PCR with Taq enzyme (#M0267 NEB, USA) and specific primers (available on request). qPCR was performed using BioMaster HS-qPCR (2×) (MN020-2040 Biolabmix, Russia) kit with specific primers and FAM-BHQ1 probe (qpcr_M2RTta_F: AGACTGGACAAGAGCAAAGT; qpcr_M2RTta_R: TTGAGCAGCCTACCCTGT; qpcr_M2RTta_probe: FAM-TCGAAGGCCTGACGACAAGGA-BHQ; qpcr_Syn1_F CCCAAATACCAGGCAACCCA, qpcr_Syn1_R GGAAGGGGCTCAACAGTAGG, qpcr_Syn1_probe: FAM-TTGGTCCCAAATCTCTCCAGCACA-BHQ). To allow absolute quantification, plasmid vector containing transposon and *SYN1* PCR fragments was constructed and used for normalization. The data was analyzed using 2^{ddCt} methods implemented in QuantStudio v1.3 software (Applied Biosistems, USA).

Double-side Inverse-PCR sequencing library preparation. DNA was isolated from cell pellet by phenol-chloroform extraction. DNA was digested overnight in 50 µl reaction at 37 °C by 5U *Nla*III isoschizomer *Fae*I (E495 SibEnzyme, Russia) in final DNA concentration 100 ng/µl. Enzyme was inactivated by incubation at 65 °C for 10 min and 500 ng of digested DNA was ligated using T4 DNA ligase (E319, SibEnzyme) at 4 °C overnight in 100 µl reaction volume. 1 µl of ligation mix was used in PCR with Taq polymerase (#M0267 NEB, USA) (annealing 60 °C, elongation 3 min, 40 cycles). PCR products were diluted 100-fold and 1 µl was used in the next round of nested PCR. PCR products were analyzed by agarose gel electrophoresis and either used in the third round of nested PCR or purified using AMPure XP beads (A63882 Beckman Coulter, USA). 1 ng of purified PCR product was used for NGS adaptor ligation (SeqCap Adapter Kit B, 07141548001 Roche, Switzerland) using KAPA HyperPrep kit (07962363001 Roshe) according to manufacturer protocol with 15 cycles of post-ligation PCR.

NGS data analysis. We demultiplexed reads originating from different NGS-libraries based on barcode sequences (barcode_seq) included in 5′-end of primers using cutadapt (Martin, 2011) with –g barcode_seq –G barcode_seq

–overlap 6 –e 0. Next, primers (primer_seq) were removed using cutadapt with parameters –g primer_seq –G primer_seq –overlap 20. Processed reads were aligned to human genome hg38 using bwa mem with default parameters (Li, Durbin, 2009). Regions covered by at least one read were found using bedtools genomecov (Quinlan, Hall, 2010) and a homemade python script. In addition, every covered region was manually analyzed in Integrated Genome Browser (IGV) (Robinson et al., 2011), which allowed distinguishing insertions sites from random ligations and other artifacts. The following analysis of rearrangements was done using homemade python scripts that counted the number of reads with mates or supplementary alignments in different insertion sites.

Results and discussion

We improved a standard inverse-PCR assay for efficient localization of transgene integration. A key aspect of this strategy is the ability to recognize simultaneously both 5′ and 3′ flanking regions of transgenes in a multiplex NGS-based assay. As in conventional inverse-PCR, our assay consists of five steps: 1) DNA fragmentation, 2) ligation under low DNA concentration conditions, which favors circularization, 3) nested-PCR using primer pairs annealing to the ends of transgenic sequence in outward orientation, 4) PCR products sequencing, and 5) computational analysis.

In our modification (Fig. 1), we propose to fragment DNA by restriction enzyme (RE), which recognizes sites that are absent in the transgene sequence. This results in the generation of DNA fragments, containing transposon and both 5′ and 3′ flanking regions up to the first RE recognition site in length. A subsequent ligation reaction generates circular molecules, which enables us to proceed with nested inverse-PCRs. PCR products are then used for NGS library preparation and paired-end sequencing. Reads are next trimmed from primers and transgenic sequences and aligned to the reference genome, which produces a recognizable pattern, defining integration sites and allowing to distinguish them from artifacts.

To prove this concept, we chose to use the pSB_LoxP plasmid previously constructed in our laboratory as a sleeping beauty (SB) transposon-containing vector. It contains a short LoxP-sequence cloned between two SB inverse terminal repeats (ITRs), and via transposase-mediated integration generates 833 bp long DNA inserts. Although 4-bp cutting RE is preferable for effective Inverse-PCR library construction, it is hardly possible to find at least one RE that does not cleave the integrating DNA. However, we noted that the transposon sequence contains a single *Fae*I (*Nla*III) recognition site within ITR sequence. To disrupt this site, we introduced single nucleotide substitution by site-directed mutagenesis. This allowed us to employ *Fae*I as RE for DNA fragmentation in integration localization assay.

To test this approach, we co-transfected human HAP1 cells with the developed transposon-containing plasmid and the SB100X vector expressing transposase and GFP proteins, followed by cell sorting of the GFP-positive cells the next day. Five days after transfection the GFP-negative cells were subcloned using FACS. This ensured the loss of the transposase-expressing plasmid and excluded the possibility of continuous "jumping" of the transposons across the genome. Two of the obtained subclones were randomly picked to proceed with localization assay.

For these clones, we constructed and sequenced an Inverse PCR NGS library following the approach described above. We obtained ~300000 read pairs for the first and ~200000 for the second clone. NGS data analysis suggested 73 and 13 integration site candidates (regions covered by at least ten reads) for each clone respectively. Every covered region was manually analyzed in IGV genome browser to distinguish insertion sites from random ligations and other artifacts. This analysis allowed us to identify 12 transposon insertions in

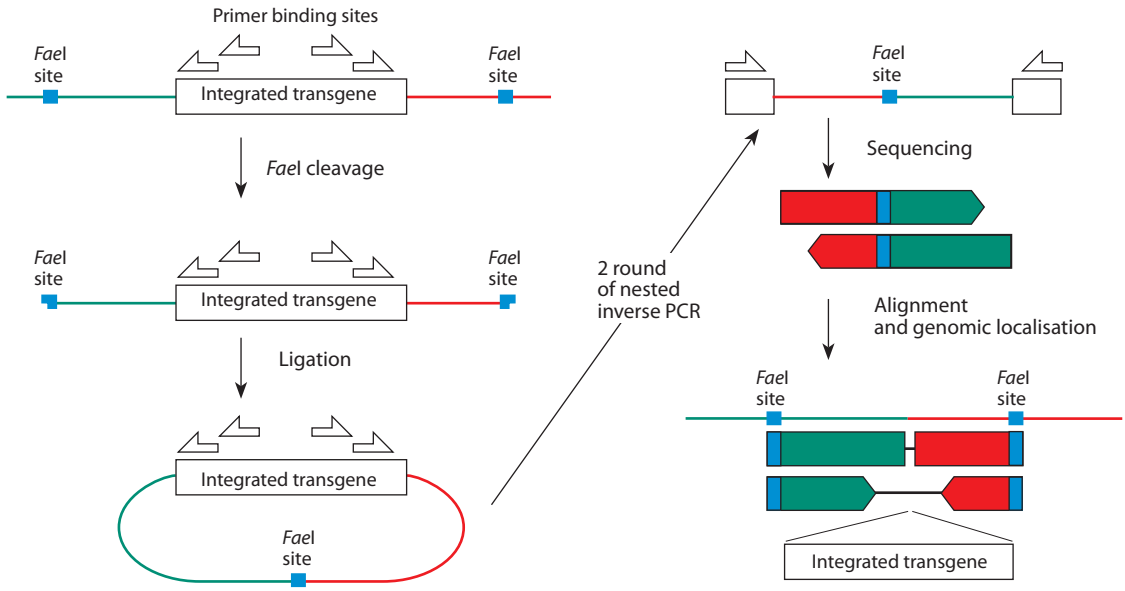

Fig. 1. Conceptual scheme of Inverse-PCR-based strategy allowing double-side detection of transgene integration sites.

Genomic DNA carrying transgene integration sequentially are fragmented, re-ligated, PCRed from nested primers and obtained products are sequenced. Resulting reads alignment forms recognizable patterns depending on integration point and fragmentation sites position.

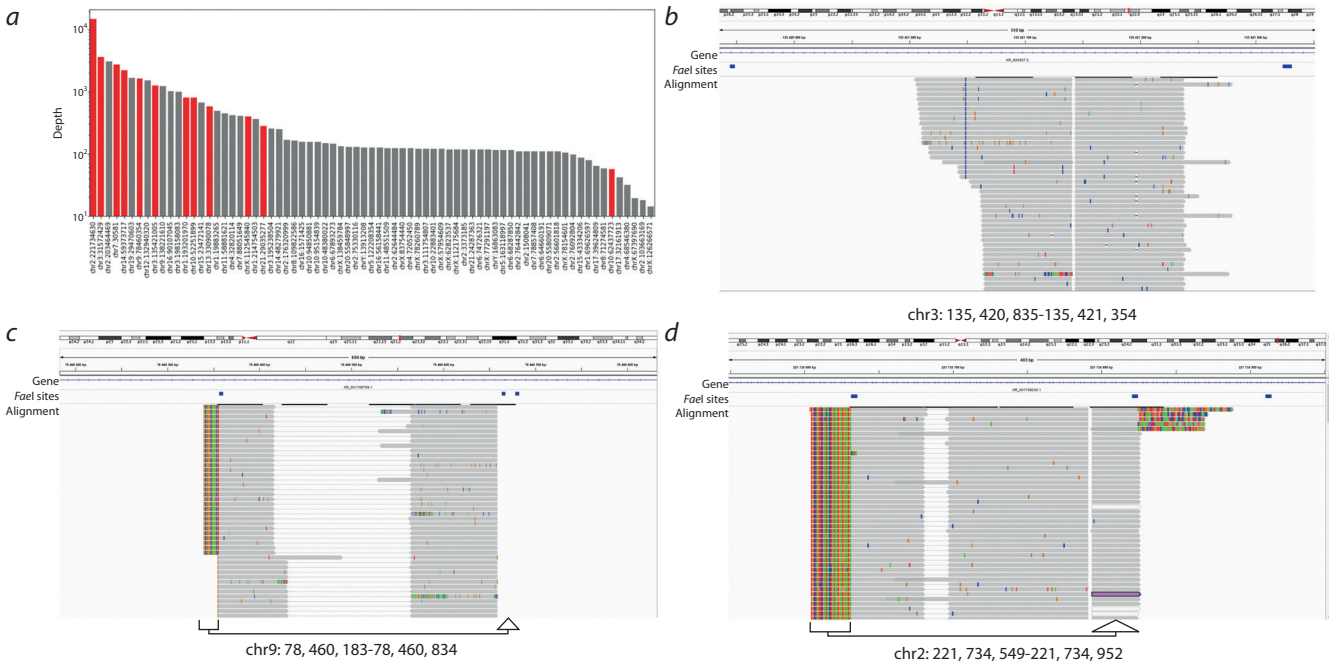

Fig. 2. Transposon integrations analysis.

a – bar plot representation of sequencing depth (*y*, log scale) among integration candidate sites (*x*) for the first subclone. Red bars represent bona fide integrations, gray – ligation artifacts. Bona fide integrations were discriminated based on the manual curation of NGS results in the IGV browser and confirmed using PCR (see the text for details); *b*–*d* – IGV screenshots showing read alignments for some integrations. Arrows underneath represent supplementary sequences or soft-clipped read bases corresponding to the bases transferred from another transgene flanking region through the *Fae*I site.

the first clone and 6 insertions in the second clone. The rest were identified as artifacts generated on ligation step and supplementary alignment regions for transposons integrated into repetitive DNA elements.

To obtain an estimate of the number of integrations using an orthogonal approach, we employed qPCR strategy with internal plasmids control (see Materials and methods). We obtained approximately 20 insertions for the first clone and 10 for the second. Despite the possibly low estimation accuracy of qPCR method, this result can identify bias of insertion under-representation of our Inverse-PCR-based strategy. Moreover, coverage is not a sufficient parameter for integration site identification. As shown on Fig. 2, *a*, both high-represented artifacts and low-represented transposon integrations are observed, although *bona fide* integration sites typically show higher coverage.

To dissect the nature of these biases, we investigated alignments individually. The typical expected pattern is shown on Fig. 2, *b*: both mates in the reads pair start at the same point (TA dinucleotide, the obligatory SB integration site) and extend divergently. Depending on RE sites position, this pattern can transform: if a read crosses the RE site involved in the DNA circularization, its alignment will be truncated at RE site position and continue again on the other side of transgene integration loci (see Fig. 2, *c*, *d*). The situation is more complicated if two RE sites are close to integration on both sides. The distance between these sites may be smaller than read length, and in this case transposon sequence appears at the end of the read. The worst case is when transposon integration is in a repeated sequence, which results in multiple reads alignment and difficulties with precise localization (Fig. 3, *a* and *b*). We observed one integration located in a repeated sequence and also flanked by closely located *Fae*I sites. We managed to recognize its position only using complementary information accidentally produced due to unintended ligation of a random sequence to one of the transgene ends. Supplementary aligned bases of those reads revealed the *bona fide* transposon integration point (see Fig. 3, *c* and *d*).

Further, we decided to validate the integration sites determined by NGS-approach using conventional PCR. Six transposons were mapped within intergenic regions, and seven were integrated into gene introns. Since SB integration is semi-random and tends to occur in the active chromatin, this is not surprising. However, the genomic context of most integration sites in our subclones is low-complex. We picked a few integrations, the flanking regions of which allow us to design PCR primers. First, we confirmed integration events from one side using primer pairs, one of which was targeted to flanking regions and the other was placed in the transposon (see Fig. 3, *e*). Next, we obtained the PCR products containing the entire transposon with flanking sequences, using pairs of primers targeted to endogenous regions around the predicted integration site. This unambiguously confirmed the NGS-based localization results (see Fig. 3, *f*).

Conclusion

Summing up, we demonstrated the applicability of the proposed double-side transgene localization approach by successfully mapping 18 SB transposon integrations carrying an exogenic sequence. Our method provides simple detection of integration sites with confident artifact filtration via analysis of the read pair alignment pattern. Because the developed methods allow simultaneous detection of the flanking sequences at both transgene ends, we argue that this method can be used

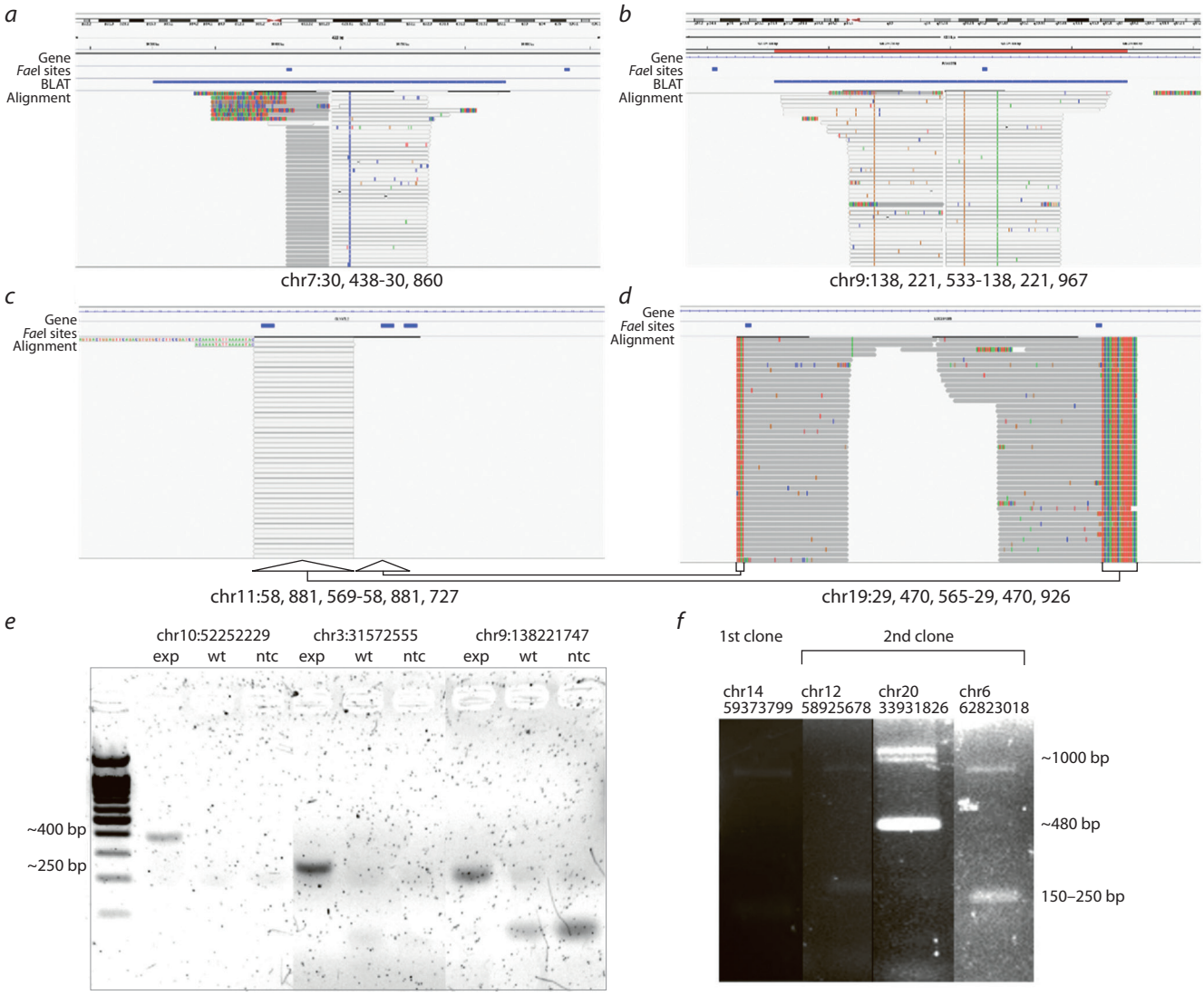

Fig. 3. Transposon integrations analysis and PCR-confirmation.

a–d – IGV screenshots showing read alignments for some integrations (*a–c*) and ligation artifact (*d*). Arrows underneath represent supplementary sequences or soft-clipped read bases, corresponding to the bases transferred from another transgene flanking region through the *Fae*I site. *e* – one side PCR detection of transposon integration in the first clone. exp – experimental subclone with transposon integrations; wt – intact HAP1 cells; ntc – non-template control; *f* – double-side PCR integration detection. 1 kb product represents allele carrying transposon integration, whereas lower bands correspond to wild type allele product.

in future for genome-wide detection of transgene recombination events. Notably, the SB-transposon derived in this study contained a LoxP-site sequence, which makes it very simple to repurpose the developed system for induction of Cre-mediated chromosomal rearrangements.

However, these experiments revealed some limitations of the developed method that we have to discuss. First, the proximity of chosen RE sites to transposon integration has a huge influence on the representation of integration site in the sequencing data. In this experiment we have already seen that too close RE sites complicate transposon mapping, whereas too distant sites can fully prevent PCR product generation. It happens because library preparation steps such as PCR and purification have a size-selecting manner. To solve this problem, one may develop new protocols where sonication is used for DNA fragmentation during library construction. Importantly, sonication will yield significantly higher noise for transgene recombination events detection due to the possibility to introduce DNA breaks inside the transgene. Second, in contrast to common inverse-PCR, our approach requires the choice of a RE that would not cut the transgene sequence. It is challenging even for short insertions, so we propose to introduce single-nucleotide substitutions into vector sequences. Third, neither our approach, nor other transgene localization methods ensure the identification of all integrations. For practical use, we recommend complementing our method with transgene insertions quantification via qPCR.

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Evaluation of developmental competence of *Sus scrofa domesticus* (L.) oocyte-cumulus complexes after intra- and extraovarian vitrification

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Abstract. The aim of the present study was to identify the influence of extra- (EOV) and intraovarian vitrification (IOV) on mitochondrial activity (MA) and chromatin state in porcine oocytes during maturation *in vitro*. During EOV porcine oocytes were exposed in cryoprotective solutions (CPS): CPS-1 – 0.7 M dimethyl sulfoxide (DMSO) + 0.9 M ethylene glycol (EG); CPS-2 – 1.4 M DMSO + 1.8 M EG; CPS-3 – 2.8 M DMSO + 3.6 M EG + 0.65 M trehalose. At IOV the ovarian fragments were exposed in CPS-1 – 7.5 % EG + 7.5 % DMSO, then in CPS-2 – 15 % EG, 15 % DMSO and 0.5 M sucrose. Straws with oocytes and ovarian fragments were plunged into LN2 and stored. For devitrification, the EOV oocytes were washed in solutions of 0.25, 0.19 and 0.125 M of trehalose, the IOV – in 0.5 and 0.25 M trehalose. Oocytes were cultured in NCSU-23 medium with 10 % fluid of follicles, follicular walls, hormones. 0.001 % of highly dispersed silica nanoparticles (ICP named after A.A. Chuyko of the NAS of Ukraine) were added to all media. The methods of fertilization and embryo culture are presented in the guidelines developed by us. MA and chromatin state were measured by MitoTracker Orange CMTMRos and the cytogenetic method. Significant differences in the level of oocytes with high-expanded cumulus between control and experimental vitrified groups (81 % versus 59 % and 52 %, respectively, $p \le 0.001$) were observed. The percentage of pyknotic cells in native oocytes was 19 %, EOV or IOV oocytes were 39 % and 49 %, respectively. After culture, the level of matured native oocytes was 86 %, 48 % EOV and 33 % IOV cells finished the maturation ($p \le 0.001$). Differences were also observed in the level of MA between groups treated by EOV and IOV (89.4 ± 7.5 μA and 149.2 ± 11.3 μA, respectively, $p \le 0.05$). For the first time, pre-implantation embryos were obtained from oocytes treated by IOV.
Key words: oocyte; vitrification; extraovarian; intraovarian; mitochondria; *Sus scrofa domesticus* (L.).

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Оценка компетентности к развитию ооцит-кумулюсных комплексов *Sus scrofa domesticus* (L.) после интра- и экстраовариальной витрификации

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Аннотация. Цель настоящей работы – идентификация влияния экстра- (ЭОВ) и интраовариальной витрификации (ИОВ) на митохондриальную активность (МА), состояния хроматина в ооцитах свиней в процессе созревания *in vitro*. При ЭОВ ооциты свиней обрабатывали растворами криопротекторов (КПР): КПР-1 – 0.7 М диметилсульфоксида (ДМСО) + 0.9 М этиленгликоля (ЭГ); КПР-2 – 1.4 М ДМСО + 1.8 М ЭГ; КПР-3 – 2.8 М ДМСО + 3.6 М ЭГ + 0.65 М трегалозы. При ИОВ фрагменты яичников опускали в КПР-1 – 7.5 % ЭГ + 7.5 % ДМСО, затем в КПР-2 – 15 % ЭГ, 15 % ДМСО и 0.5 М сахарозы. Пайеты с ооцитами и фрагменты яичников погружали и хранили в LN_2. Для девитрификации ЭОВ ооциты экспонировали в 0.25, 0.19 и 0.125 М растворах трегалозы, ИОВ – в 0.5 и 0.25 М трегалозы. Ооциты культивировали в среде NCSU-23 с 10 % жидкости фолликулов, их стенками, гормонами. Все среды дополняли 0.001 % наночастиц высокодисперсного кремнезема (Институт химии поверхности им. А.А. Чуйко Национальной академии наук Украины, Украина). Режимы оплодотворения и культивирования эмбрионов представлены нами в методических рекомендациях. Митохондриальную активность и статус хроматина оценивали MitoTracker Orange CMTMRos и цитогенетическим методом. Выявлены достоверные различия в уровне ооцитов с высокоэкспандированным кумулюсом между контрольной

и витрифицированными группами (81 % против 59 и 52 % соответственно, $p \leq 0.001$). Доля пикнотических клеток у нативных ооцитов составила 19 %, у ЭОВ и ИОВ ооцитов – 39 и 49 % соответственно. Стадии метафазы II достигли 86 % нативных ооцитов, и только 48 % ЭОВ и 33 % ИОВ ооцитов завершили созревание ($p \leq 0.001$). Отмечена достоверная разница в МА между группами, подвергнутыми ИОВ и ЭОВ (89.4 ± 7.5 и 149.2 ± 11.3 мкА соответственно, $p < 0.05$). Впервые получены доимплантационные эмбрионы из ооцитов свиней, подвергнутых интраовариальной витрификации.
Ключевые слова: ооцит; витрификация; экстраовариальная; интраовариальная; митохондрии; *Sus scrofa domesticus* (L.).

Introduction

The development of a vitrification method for cryopreservation of reproductive cells is the most significant achievement for human and animal ART over the past 70 years (Coello et al., 2018). However, after more than half a century of research in this area, the results for production of viable embryos from devitrified oocytes remain controversial (Mullen, Fahy, 2012). Firstly, this is closely related to the slow-growing progress in upgrading the protocols (parameters) of extra- or intraovarian freezing/thawing technology (Yurchuk et al., 2018).

In case of extraovarian vitrification of female gametes using open freezing systems, such as straws, cryotopes, cryolopes, the saturation of cells by cryoprotectants can be achieved in a short time with a relatively short exposure time in vitrification solutions, as well as the transition of cells to a vitrified state; in case of a closed intraovarian (intrafollicular) system, the exposure time in cryoprotectant solutions significantly increases, and the rate of transition of intracellular water to the "glass-like" phase is slower due to an increase in the eutectic point (Obata et al., 2018). Due to the lengthening of the water phase transition, there is a danger of the formation of extra- and intracellular ice crystals, which have a damaging effect on cells (Amstislavsky et al., 2015). However, when using an open method of vitrification, there is a risk of invasion of the vitrification medium and oocyte-cumulus complexes, which can subsequently affect the competence of cells for fertilization and subsequent embryo development (Joaquim et al., 2017). The intraovarian vitrification can become an alternative closed system, which eliminates the damaging effect of resistant cryogenic microorganisms and fungi on ovarian tissue and oocytes (Bielanski, 2012). Meanwhile, the usage of both vitrification models implies the occurrence of temperature- and osmotically-dependent damage to the subcellular compartments of germ and somatic cells (Buderatska, Petrushko, 2016).

The most sensitive organelles are the cytoskeleton, mitochondria, and the nuclear apparatus, which play an important role in the proliferation of somatic cells, as well as the maturation and further development of female gametes (Lai et al., 2014). As a consequence of cryogenic phase-structural transitions and peroxidation of annular lipids, the barrier properties of the mitochondrial membrane are disrupted, there is a leakage of transported ions, including Ca^{2+} and H^{+}, and metabolites both through the active transport and the passive diffusion via the transmembrane defects (non-specific pores with high permeability), which causes a decrease in the energy supply of the oocyte during development and contributes to triggering apoptosis (Kuzmina et al., 2019). Low-temperature damage to the nuclear apparatus of oocytes is characterized mainly by a decrease in their matrix activity (synthesis of DNA and RNA) due to the cryodenaturation and the loss of enzyme functional activity (Pereira et al., 2019).

Thus, the creation of an optimal and efficient vitrification technology, which would be able to preserve the architectonics and functional activity of cell compartments that ensure the formation of egg competent for fertilization, is one of the main challenges facing reproductive biologists and cryobiologists dealing with the low-temperature preservation of gametes.

The aim of this study is to identify the effect of various models (extra- and intraovarian) of vitrification on the functional activity of mitochondria (fluorescence intensity of MitoTracker Orange CMTMRos) and chromatin status in native and devitrified oocytes *Sus scrofa domesticus* (L.) during the extracorporeal maturation and the development of preimplantation embryos.

Materials and methods

All reagents used in the experiments, except those indicated in the text, were manufactured by Sigma-Aldrich (USA). Plastic laboratory glassware was from BD Falcon™ (USA).

In the experiments, the oocyte-cumulus complexes (OCC) aspirated from the ovarian antral follicles of *S. scrofa domesticus* (L.) (domestic pig) of Landrace breed were used. After ovariectomy, the porcine ovaries were delivered to the laboratory in 0.9 % NaCl solution at a temperature of 30–35 °C, containing antibiotics. For the experiments, we used oocytes surrounded by tightly packed layers of cumulus cells (more than five layers), with a uniform zona pellucida, and homogeneous ooplasm. Denuded oocytes and oocytes with loose cumulus were not used.

Cells intended for extraovarian vitrification were treated with three cryoprotectant solutions (CPA) prepared on the basis of TC-199 medium supplemented with 10 % fetal bovine serum (FBS, HyClone, UK): CPA-1 – 0.7 M dimethyl sulfoxide (DMSO) + 0.9 M ethylene glycol (EG); CPA-2 – 1.4 M DMSO + 1.8 M EG; CPA-3 – 2.8 M DMSO + 3.6 M EG + 0.65 M trehalose. Oocyte-cumulus complexes was gradually exposed for 30 sec in CPA-1, then 30 sec in CPA-2 and 20 sec in CPA-3. During intraovarian vitrification, the dissected ovaries were divided into 6–8 sections (15 × 20 mm), placed in sterile gauze bags and dipped in CPA solutions based on Dulbecco's phosphate buffer solution (PBS) with the addition of 20 % FBS: CPA-1 – 7.5 % EG + 7.5 % DMSO (15 min), then in CPA-2 – 15 % EG, 15 % DMSO and 0.5 M sucrose (2 min). Straws with oocytes and sterile bags with ovarian fragments were immersed in LN_2 (−196 °C) for at least 1 h. Extraovarially vitrified OCCs were removed from the straws after thawing and exposed in 0.25 M trehalose solution

(3 min) based on medium TC-199 with the supplementation of 10 % FBS at 37 °C, were sequentially washed in a 0.19 M solution (3 min) and then in a 0.125 M solution of trehalose (3 min). Aspirated oocytes from fragments, after thawing, were sequentially treated with 0.5 M (1 min) and 0.25 M (5 min) trehalose solutions prepared on the basis of PBS with 20 % FBS content. The final washing of cells was carried out in TC-199 medium with 10 % FBS. All vitrification/devitrification media were supplemented by highly dispersed silica nanoparticles (nHDS) at a concentration of 0.001 % (Chuiko Institute of Surface Chemistry of National Academy of Sciences of Ukraine, Ukraine). The concentration used in the experiments was chosen according to the data obtained by the developers (Galagan et al., 2010).

Native and devitrified OCCs were cultured in an atmosphere with 5 % CO_2 at 90 % humidity, a temperature of 38 °C, in North Carolina State University-23 (NCSU-23) medium with 10 % follicular fluid (from follicles with a diameter of 3–6 mm), 10 M.E. human chorionic gonadotropin, 10 M.E. horse chorionic gonadotropin, fragments of follicular walls (600 × 900 μm), 50 μg/ml gentamicin and 0.001 % HDS nanoparticles (Abeydeera et al., 1998). The protocols of oocyte fertilization and embryo culture are presented in the guidelines (Kuzmina et al., 2008).

For assessing mitochondrial activity in native and devitrified oocytes, a MitoTracker Orange CMTMRos fluorescent probe (Thermofisher Scientific, UK) was used. Oocyte-cumulus complexes was placed into drops of 500 nM solution and incubated in the dark at 37 °C for 30 min. OCCs were washed in PBS with the addition of 0.3 % bovine serum albumin. The washed oocytes were denuded from cumulus cells by incubation in 0.1 % trypsin solution at 37 °C for 5–10 min, transferred into Hanks solution containing 3.7 % paraformaldehyde, and fixed (15 min, 37 °C). After fixation, oocytes were washed in PBS and placed on Superfrost slides.

To analyze the chromatin state, denuded (from cumulus) oocytes and cumulus cells were placed for 5–10 min in a warm 0.9 % hypotonic solution of sodium citrate 3-substituted. Then the cells were fixed on the slides with a mixture of methanol and acetic acid (3:1). Dry-air slides were stained with 4 % Romanovsky–Giemsa solution for 3–4 min (Tarkowski, 1966).

The MitoTracker Orange CMTMRos fluorescence intensity measurement and assessment of nuclear maturation in native and devitrified oocytes, the level of pyknosis in cumulus cells were performed using a fluorescent microscope Axio Imager A2 (Carl Zeiss, Germany) and a photometer (Nikon, Germany). Excitation wavelengths for MitoTracker Orange CMTMRos – 554 nm, radiation – 576 nm. The fluorescence intensity of MitoTracker Orange CMTMRos was measured in μA.

Statistical analysis of the results was carried out using SigmaStat statistical program (Jandel Scientific Software, USA). Results of the present study are predominantly presented using descriptive statistics. Data are presented as means (M) and standard errors (±SEM), as well as frequency variables. To assess the significance of differences between the values, Student's t-test and Pearson's χ^2 test were used. Results were considered significant when $p < 0.05$, $p < 0.01$, $p < 0.001$.

Results and discussion

Difficulties in the development of effective oocyte freezing method are primarily associated with the structural and functional features of the egg organization, as well as the intra- and intercellular signal interactions in devitrified oocytes (Moussa et al., 2014).

In our studies, it was revealed that the proportion of oocytes surrounded by highly-expanded cumulus in the control group significantly exceeded those in devitrified groups, regardless of the vitrification model (81 % versus 59 and 52 %, respectively, $p \leq 0.001$) (Fig. 1 and 2). There were no significant differences between the groups of oocytes vitrified outside (extra-) or inside (intra-) fragments of the ovary (see Fig. 1). Analysis of destructive processes in cumulus cells of native and devitrified oocytes showed significant differences in the level of pyknosis between all experimental groups (see Fig. 1). It was found that the native control group had the smallest percentage of chromatin destruction of cumulus cells (19 %). No significant differences were noted in the level of cumulus cells with pyknotic nuclei surrounding extra- and intraovarially devitrified oocytes (39 and 49 %, respectively).

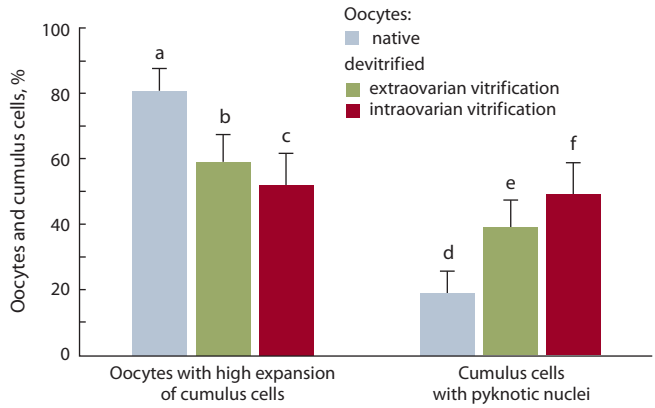

Fig. 1. Analysis of cryoresistance indicators of follicular somatic cells (cumulus) of *S. scrofa domesticus* (L.) with the use of different vitrification models (intra- and extraovarian, number of oocytes – 379, number of experiments – 3).

Differences are statistically significant (χ^2-test): a:b; a:c; d:f $p \leq 0.001$; d:e $p \leq 0.01$.

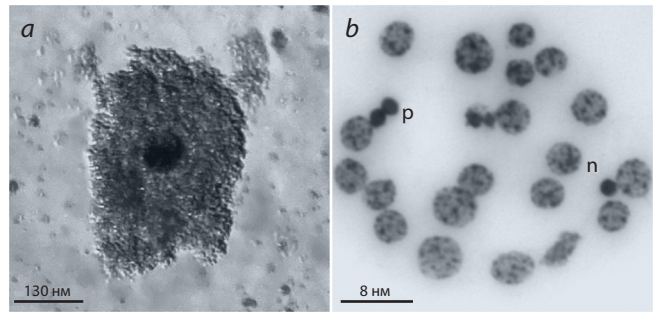

Fig. 2. Oocyte-cumulus complex with high cumulus cell expansion (*a*) and cumulus cells of *S. scrofa domesticus* (L.) with normal, n, and pyknotic, p nuclei (*b*) after extraovarian vitrification.

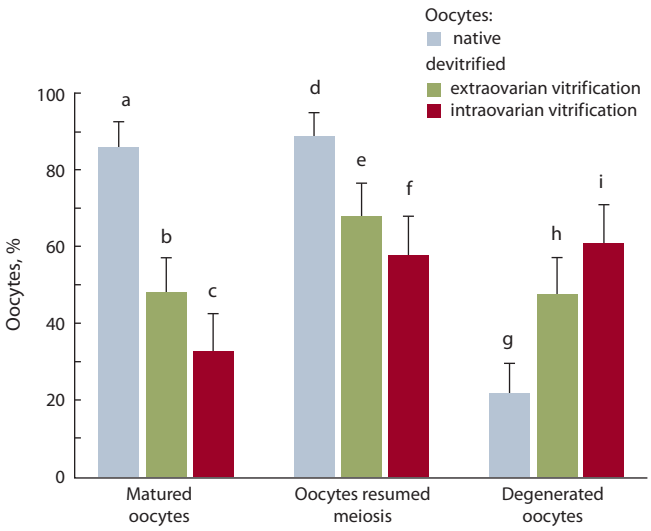

Fig. 3. Chromatin status of native and devitrified porcine oocytes after *in vitro* culture with the use of different vitrification models (intra- and extraovarian, number of oocytes – 323, number of experiments – 3).

Differences are statistically significant (χ^2-test): $^{a:b; a:c; d:e; d:f; g:h; g:i}p \le 0.001$; $^{b:c}p \le 0.05$.

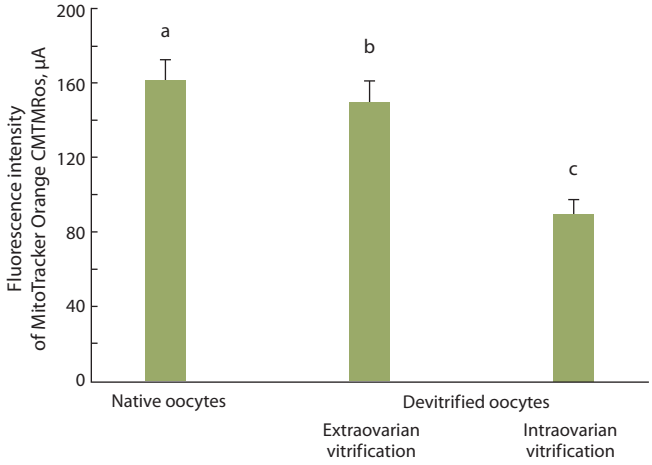

Fig. 5. Fluorescence intensity of MitoTracker Orange CMTMRos in native and divitrified oocytes of *S. scrofa domesticus* (L.) (M ± SEM, number of oocytes – 103, number of experiments – 3).

Differences are statistically significant (Student-test): $^{a:c; b:c}p \le 0.05$.

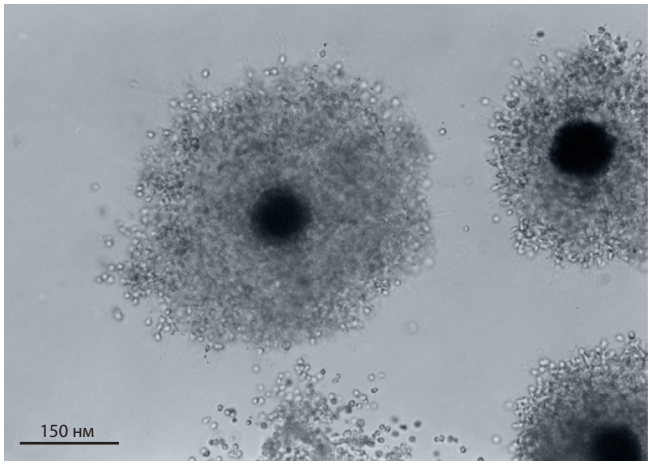

Fig. 4. Intraovarially vitrified oocytes of *S. scrofa domesticus* (L.) after 44 h of *in vitro* culture.

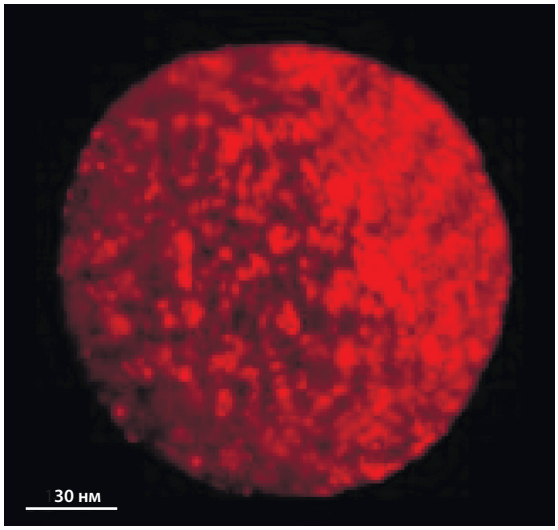

Fig. 6. Oocyte of *S. scrofa domesticus* (L.) with high level of MitoTracker Orange CMTMRos fluorescence intensity after extraovarian vitrification.

During *in vitro* culture of oocytes, it was demonstrated that 68 % of the extraovarilly vitrified cells reinitiated meiosis; in case of intraovarian vitrification, this rate was 58 %, which was significantly lower than that in the native control group (89 %, $p \le 0.001$) (Fig. 3 and 4). About a half of oocytes after extraovarian vitrification (49 %) reached the final maturation stage (metaphase II), the proportion of matured cells previously vitrified within follicles was 33 %, while the percentage of native cells that completed their maturation was 86 % ($p \le 0.001$). The percentage of cells with the chromatin destruction among native oocytes reached 22 % vs. 48 % and 61 % among extra-/intraovarially vitrified porcine oocytes, respectively ($p \le 0.001$).

Mitochondria provide the cell with ATP which is necessary for completion of meiosis, and the features of their functioning are one of the biomarkers of functional state and gamete quality (Al-Zubaidi et al., 2019). It was noted that the mitochondrial potential of intraovarially vitrified oocytes (fluorescence intensity of MitoTracker Orange CMTMRos) was significantly reduced compared to oocytes vitrified outside of the follicles (89.4 ± 7.5 versus 149.2 ± 11.3 μA, respectively, $p \le 0.05$) (Fig. 5 and 6). In the native group of oocytes, the MitoTracker Orange CMTMRos fluorescence intensity was 161.2 ± 10.8 μA.

Embryos at the final stage of pre-implantation development (the blastocyst stage) were obtained in all experimental groups (Fig. 7 and 8). After fertilization of the experimental groups of oocytes (extra- and intraovarially vitrified), the cleavage rates amounting to 27 and 21 %, respectively, were discovered to be lower than in intact native cells (49 %, $p \le 0.001$). The

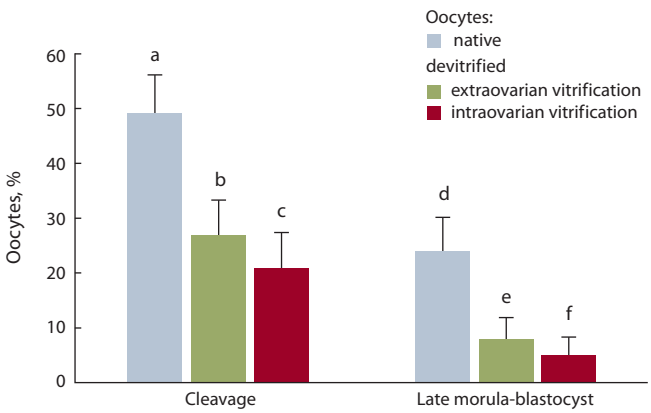

Fig. 7. Development of pre-implantation embryos of *S. scrofa domesticus* (L.) obtained from devitrified oocytes (number of oocytes – 556, number of experiments – 3).

Differences are statistically significant (χ^2-test): $^{a:c; d:f}$ $p \le 0.001$; $^{a:b; d:e}$ $p \le 0.01$.

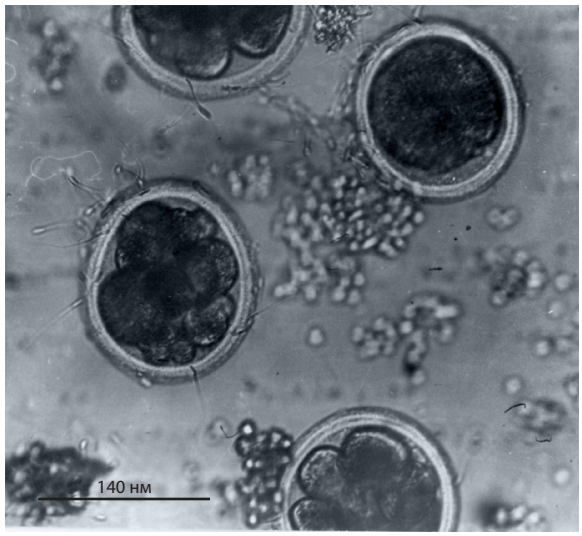

Fig. 8. Cleavage embryos obtained from extraovarialy vitrified oocytes of *S. scrofa domesticus* (L.).

yield of embryos at late morula, blastocyst stages, obtained from oocytes, vitrified intra- and extraovarially was 5 and 8 %, respectively.

The main indirect indicator by which one can judge the maturity and development competence of the oocyte is the degree of cumulus expansion (Spricigo et al., 2011). According to our analysis of the degree of cumulus expansion after 44 h of culture of native and devitrified oocytes of *S. scrofa domesticus* (L.), it was demonstrated that the largest proportion of oocytes with a low cumulus expansion was detected among extra- or intraovarially vitrified cells compared to the intact control group (59 and 52 % versus 86 %, $p \le 0.001$). Ultra-low temperatures cause the decrease in the expansion cumulus of porcine oocytes due to the damage of structures called "transzonal bridges" formed by gap junctions and communicating via paracrine signals (Appeltant et al., 2017). The growth of the pyknotic process of the nuclei of devitrified cumulus cells can be explained by the excessive chromosome condensation during the process of cryopreservation (cell dehydration during exposure in cryoprotectant solutions), leading to the "wrinkling" of the cell nucleus, which causes a reduction in the number of normally functioning cumulus cells (Wei et al., 2016; Kokotsaki et al., 2018).

Cumulus cells provide the oocyte with cyclic guanosine monophosphate, which prevents the destruction of cAMP by inhibiting its hydrolysis by PDE3A phosphodiesterase (Mehlmann, 2005), and thus supports the arrest of the first meiotic division at the prophase I stage. With a subsequent decrease in the cAMP level and activation of MPF (maturation promoting factor) due to dephosphorylation of *p34cdc2* and the synthesis of cyclin B, the reinitiation of meiosis is stimulated (Yang et al., 2010). Our studies have shown that both models of vitrification (extra- or intraovarian) promote the inhibition of meiosis reinitiation in more than a half of devitrified oocytes (see Fig. 2) due to a thermo-dependent rupture of the communication between cumulus cells and oocyte and, as a consequence, a violation of the concentration balance of intracellular cAMP (Mehlmann, 2005).

During culture of extra- or intraovarially vitrified oocytes, the proportion of matured oocytes with normal chromatin sharply decreases, the level of cells with meiotic aberrations increases compared to the control group, owning to the destruction of nucleotides, the appearance of DNA single-/double-stranded breaks (Pereira et al., 2019). An increase in the number of degenerated cells during *in vitro* culture can be associated with the violation of processes of spindle polymerization/depolymerization during oocyte nuclear maturation (metaphase I–anaphase stage) and subsequent disruption of its assembly, which affects chromosome segregation during the first meiotic division (Yang et al., 2012).

During ultra-low temperature cooling of oocyte, mitochondria are exposed to an excessive load of the ionized form of Ca^{2+}, due to an increase in the cytosolic concentration of Ca^{2+} in the cell (Shahsavari et al., 2019). Due to such load, non-specific high permeability pores are opened, which leads to the death of oocytes by the apoptotic mechanism (Novoderezhkina et al., 2016). The oxidative stress, mediated by the accumulation of reactive oxygen species, plays the main role in the opening of non-specific pores during freezing, which causes the destruction of membrane proteins and a reduction of the mitochondrial transmembrane potential (Zavodnik, 2016).

Thus, a decrease in the mitochondrial potential during vitrification may be associated with a shift in the concentration of reactive oxygen species, and, as a consequence, an increase in intracellular Ca^{2+} concentration and the opening of non-specific pores. A significant decrease in mitochondrial functional activity of intraovarially vitrified group of oocytes as compared to extraovarially vitrified oocytes may be caused by additional processes of tissue recrystallization due to insufficient saturation of ovarian tissues with cryoprotectants (Kuzmina, Chistyakova, 2020).

A decrease in the cleavage and embryo yield from intra-/extraovarially vitrified oocytes is possibly associated with the temporary increase in the intracellular concentration of Ca^{2+}

in gametes during exposure to cryoprotectants and cooling (Larman et al., 2006), which leads to the exocytosis of cortical granules (Kline D., Kline J.T., 1992) and premature hardening of the zona pellucida, which prevents fertilization of the egg.

Conclusion

Cryobanks, as sources of biological raw materials, are of a great importance for the subsequent use of mammalian oocytes or their ooplasts in cellular and genetic engineering, in particular, the CRISPR-cas9 genomic editing technique, as well as in preserving the gene pool of endangered breeds and genetic diversity. The development of an effective vitrification procedure through different approaches, including the usage of substances of various (natural or synthetic) origin with cryoprotective properties, is the main practical line of development of reproductive biology.

In our work, we analyzed the indicators of nuclear-cytoplasmic maturation of donor porcine oocytes exposed to ultra-low temperatures, including the chromatin state and level of oocyte mitochondrial activity. The revealed features in the functioning of the indicated cell compartments would complement the available data on the nature of destructive processes caused by vitrification/devitrification procedures. Exposure to ultra-low temperatures promoted a decrease in the level of oocytes that completed nuclear maturation and a decrease in MitoTracker Orange CMTMRos fluorescence intensity (a marker of mitochondrial functional activity).

The work also showed the importance of communication between the oocyte and the surrounding somatic cells of the ovarian follicle (cumulus). The cumulus cell morphology after the cryopreservation procedure (vitrification/devitrification) largely determined the "fate" of the oocyte – the completion of nuclear maturation (reaching of the metaphase II stage by the oocyte) and the functional activity of mitochondria. The presented protocols of intra- and extraovarian vitrification/devitrification, improved by the addition of highly dispersed silica nanoparticles to cryoprotective solutions and culture media, have provided pre-implantation porcine embryos (*S. scrofa domesticus* (L.)) from oocytes of devitrified ovarian tissue for the first time.

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Genetic resources of narrow-leaved lupine (*Lupinus angustifolius* L.) and their role in its domestication and breeding

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Abstract. Narrow-leaved lupine (*Lupinus angustifolius* L.) is a cultivated multipurpose species with a very short history of domestication. It is used as a green manure, and for feed and food. This crop shows good prospects for use in pharmacology and as a source of fish feeds in aquaculture. However, its genetic potential for the development of productive and adaptable cultivars is far from being realized. For crop species, the genetic base of the cultivated gene pool has repeatedly been shown as being much narrower than that of the wild gene pool. Therefore, efficient utilization of a species' genetic resources is important for the crop's further improvement. Analyzing the information on the germplasm collections preserved in national gene banks can help perceive the worldwide diversity of *L. angustifolius* genetic resources and understand how they are studied and used. In this context, the data on the narrow-leaved lupine collection held by VIR are presented: its size and composition, the breeding status of accessions, methods of studying and disclosing intraspecific differentiation, the classifications used, and the comparison of this information with available data on other collections. It appeared that VIR's collection of narrow-leaved lupine, ranking as the world's second largest, differed significantly from others by the prevalence of advanced cultivars and breeding material in it, while wild accessions prevailed in most collections. The importance of the wild gene pool for the narrow-leaved lupine breeding in Australia, the world leader in lupine production, is highlighted. The need to get an insight into the species' ecogeographic diversity in order to develop cultivars adaptable to certain cultivation conditions is shown. The data on the testing of VIR's collection for main crop characters valuable for breeders are presented. Special attention is paid to the study of accessions with limited branching as a promising gene pool for cultivation in relatively northern regions of Russia. They demonstrate lower but more stable productivity, and suitability for cultivation in planting patterns, which has a number of agronomic advantages. Analyzing the work with narrow-leaved lupine genetic resources in different national gene banks over the world helps shape the prospects of further activities with VIR's collection as the only source of promising material for domestic breeding.
Key words: narrow-leaved lupine; genetic resources; *ex situ* collections; diversity; gene pool; intraspecific differentiation; wild forms.

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Генетические ресурсы люпина узколистного (*Lupinus angustifolius* L.) и их роль в доместикации и селекции культуры

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Аннотация. Люпин узколистный (*Lupinus angustifolius* L.) – окультуренный вид многоцелевого назначения с очень короткой историей доместикации. Его используют как сидеральную, кормовую, продовольственную культуру, в качестве корма в рыбоводстве и в фармакологии. Однако генетический потенциал вида для создания продуктивных и адаптивных сортов далеко не реализован. Неоднократно показана узкая генетическая основа окультуренного генофонда по сравнению с диким. Поэтому эффективное использование генетических ресурсов вида имеет важное значение для дальнейшего развития культуры. Разнообразие генетических ресурсов люпина узколистного в мире, степень их изученности и пути применения можно представить посредством анализа сведений о коллекциях гермоплазмы вида, сохраняемых в национальных генбанках разных стран. В контексте этого анализа в статье приведены сведения о коллекции люпина узколистного ВИР: ее чис-

ленности, составе, селекционном статусе образцов, методах изучения и выявления внутривидовой дифференциации, используемых классификациях. Показано, что коллекция люпина узколистного ВИР, занимающая второе место в мире по числу образцов, значительно отличается от других преобладанием в ней сортов научной селекции и селекционного материала, в то время как в большинстве коллекций превалируют дикие формы. Освещено значение дикого генофонда в селекции люпина узколистного в Австралии – мировом лидере производства культуры. Показана необходимость выявления эколого-географического разнообразия вида для создания сортов с адаптивными свойствами, соответствующими определенным условиям возделывания. Приведены данные оценки образцов коллекции ВИР по основным селекционно значимым признакам. Особое внимание уделено изучению образцов с ограниченным ветвлением, как перспективному генофонду для возделывания в сравнительно северных районах нашей страны. Они обладают меньшей, но более стабильной продуктивностью, пригодны для возделывания в загущенном посеве, что имеет целый ряд агротехнических преимуществ. Анализ работы с генетическими ресурсами люпина узколистного в различных национальных коллекциях мира способствует определению путей дальнейшей работы с коллекцией ВИР как единственным источником исходного материала для отечественной селекции.
Ключевые слова: люпин узколистный; генетические ресурсы; коллекции *ex situ*; разнообразие; генофонд; внутривидовая дифференциация; дикие формы.

Introduction

Collections of plant genetic resources (PGR) are germplasm repositories maintained in many countries of the world. They preserve the global diversity of cultivated plants and their wild relatives, and differ from each other in age, number of preserved accessions, taxonomic diversity, mission, and purpose. Using these criteria to compare different PGR collections is not an easy task due to the absence of an integrated documentary source containing data on all the world's plant germplasm holdings.

The genus *Lupinus* L. has a wide range of distribution. Species of the Old World (subgen. *Lupinus* L.) are widespread in the Mediterranean region and North Africa, while the New World lupines (subgen. *Platycarpos* (Wats.) Kurl.) occur in both Americas over a fairly wide range of latitudes and altitudes. Among the rich specific diversity of the genus, only a few species have been domesticated and widely introduced into agricultural production.

The most comprehensive information on lupine collections, albeit far from complete, is the European Central *Lupinus* Database (DB). It contains data of 13,964 *Lupinus* L. accessions held in 13 genebanks of 10 countries.

Regrettably, the said European DB does not contain information about the collections of VIR and Belarus; moreover, the data presented there were last updated almost ten years ago. Other sources of information on the specific composition of the world's lupine germplasm collections operate with either the same data (Święcicki et al., 2015) or even older figures (Buirchell, Cowling, 1998), or present only the total number of accessions for different species of the genus (Berger et al., 2013).

Currently, narrow-leaved lupine (*L. angustifolius* L.), also known as blue lupine, is the world's leader in the area of cultivation among other cultivated *Lupinus* spp. It is the earliest and most plastic cultivated species and the only one adapted to relatively northern latitudes. Uses of this crop are very diverse. Traditionally, this is a green manure and forage crop. Of late, it has been intensively studied and used as a food crop. Quinolizidine alkaloids in seeds of different *Lupinus* spp. are of interest for pharmacology (Vishnyakova et al., 2020). Processed grains of various lupines, including the narrow-leaved one, have been used for several decades in fish farming to prepare feeds. This aspect of lupine utilization is promoted by numerous publications and web resources. One of the examples is the summary by B.D. Glencross (2001).

Nutritive value of narrow-leaved lupine is determined by its high content of protein (30–40 %), carbohydrates (40 %), oil (6 %), numerous minerals, vitamins, and other health-friendly ingredients. It is widely cultivated in Northern and Eastern Europe (Germany, the Netherlands, Poland, etc.), the United States, New Zealand, and Belarus. The world's leader in the production and export of this crop, research into its genetic diversity, and most significant breeding achievements is Australia (Cowling, 2020; Vishnyakova et al., 2020). As far as the Russian Federation is concerned, the area of narrow-leaved lupine cultivation in 2019 reached 78,971 hectares, making this country one of the leading producers of this crop in the world (http://www.fao.org/faostat/en/#data/QC).

There are 27 cultivars listed in the State Register for Selection Achievements Admitted for Usage in Russia. Only seven of them were released in the past five years. This is not much on a national scale, considering the size of the country, but all of these cultivars were developed by Russian breeders. Intensification of plant breeding efforts obviously requires well-characterized source material. The only holder of such material in Russia is the global PGR collection of VIR.

The purpose of this publication is to analyze the worldwide diversity of narrow-leaved lupine GR preserved in national *ex situ* collections of different countries, with an emphasis on VIR's collection, and discuss the prospects for effective utilization of these resources.

When and where was *L. angustifolius* domesticated?

Lupinus angustifolius L. is a very polymorphic species with high adaptive potential. The geographic range of narrow-leaved lupine cultivation stretches from 30° S up to 60° N. Narrow-leaved lupine plants can tolerate temperatures as low as –9 °C (Kuptsov, Takunov, 2006). The known maximum altitude where this species occurs is 1,800 m above sea level. The soil pH gradient is 4.2–9.0. Annual precipitation in the areas where representatives of this species naturally occur is 200 to 1500 mm (Buirchell, Cowling, 1998). Narrow-leaved lupine plants are capable of growing on soils deficient in nitrogen

and phosphorus. The diversity of morphological characters and adaptive properties of lupine has been triggered by an extensive environmental diversity of its habitats.

The center of origin for narrow-leaved lupine is the Mediterranean region. In the wild, *L. angustifolius* occurs much more frequently than other lupine species of the Old World and is still distributed throughout the Mediterranean (Cowling, 1986) as well as in Asia Minor, Transcaucasia, and Iran (Gladstones et al., 1998). Recent studies have shown that the greatest genetic diversity is found among the wild forms of narrow-leaved lupine in the western Mediterranean. There is a suggestion that it was the gene pool of the Iberian Peninsula that migrated eastwards, thus initiating the domestication of the species (Mousavi-Derazmahalleh et al., 2018a, b).

The process of domestication for narrow-leaved lupine presumably started in the 1930–1940s, when the discovered alkaloid-free mutants (Sengbusch, 1931) enabled plant breeders in Germany and Sweden to develop the first forage cultivars (Maysuryan, Atabekova, 1974). Australian scientists, however, date it back to the 1960s and 1970s (Gladstones, 1970). At that time, plant breeders in Australia launched the development of cultivars combining in their genotype the maximum number of genes that determined the domestication syndrome, namely those controlling the absence of alkaloids (*iuc*), nonshattering of pods (*le* and *ta*), early flowering (*Jul* and *Ku*), permeability of the seed coat (*moll*), and white color of flowers and seeds (*leuc*) (Cowling, 2020). Breeding achievements in Australia led to a two to three times increase in the crop's yield in the early 21st century across the main lupine cultivation areas in Western Australia since the release of the first cultivar in 1967 (French, Buirchell, 2005).

History of the narrow-leaved lupine collection at VIR

Since N.I. Vavilov's times, VIR has been accumulating in its collection representatives of the cultivated flora together with accompanying wild relatives: species and varieties with certain individual properties that may be required and can be used by domestic plant breeders (Vavilov, 1925).

The first accessions of narrow-leaved lupine were donated to VIR in 1919 (Fig. 1) by Prof. D.N. Pryanishnikov from Moscow Agricultural Institute (now Russian State Agrarian University – Timiryazev Moscow Agricultural Academy). The lupine collection, like most of the Institute's collections, started to grow much more intensively with the coming of Nikolai Vavilov to the Institute. In the 1920s, N.I. Vavilov and the staff of VIR were busy ordering large-scale shipments of plant germplasm from various botanical gardens of France, England, Sweden, Poland, Czechoslovakia, Switzerland, Denmark, etc. Breeding material was actively procured from France (Vilmorin), England (Suttons Seeds & Bulbs) and Germany (Haage und Schmidt). Of crucial importance were Vavilov's collecting missions to the centers of lupine origin. Valuable samples of *L. angustifolius* were collected in 1926–1927 in the Mediterranean countries: Italy, Greece, Spain, Algeria, and Palestine. For example, the accessions of Palestinian origin were characterized by earliness, rapid growth in the first half of the growing season, high yield of green biomass, and high protein and oil content in seeds (Kurlovich et al., 1991). The

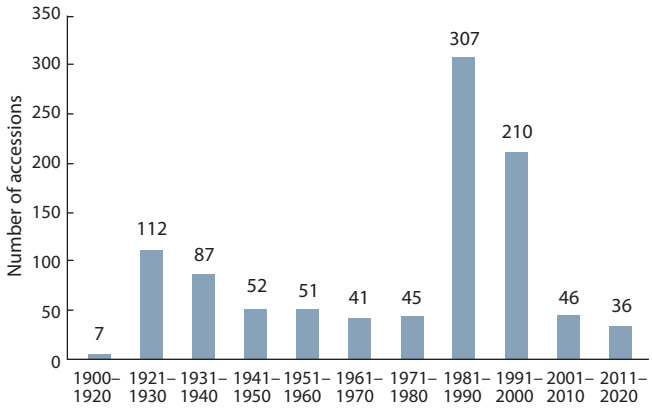

Fig. 1. Historical dynamics of adding narrow-leaved lupine accessions to the collection.

accessions from Algeria were distinguished for their thermal neutrality and high productivity. Altogether, Vavilov enriched the collection with 109 accessions of different *Lupinus* spp.: 54 from the Mediterranean countries, 9 from both Americas, and 46 from Western Ukraine and Belarus. There were 12 accessions of *L. angustifolius* among them. During his last expedition to Western Ukraine, Vavilov found an alkaloid-free sample of narrow-leaved lupine; it was added to the collection on November 16, 1940.

In the 1930s and 1940s, the collection continued to be actively expanded at the expense of, *inter alia*, the material from experiment and breeding stations, e. g., Novozybkov Experiment Station, where the breeding work with lupine began in 1925, and seed samples (for the most part, local varieties and breeding material) from Belarus and Ukraine, obtained by collecting teams during plant explorations.

During the war against the Nazi occupants, some of the preserved germplasm was lost, despite the efforts of VIR's staff to save the collection. Already in 1945, however, new seed material started to arrive from Novozybkov, Belarusian and Tiraine (Latvia) experiment and breeding stations, and from the Timiryazev Moscow Agricultural Academy.

In the following years, the collection received a lot of breeding material from Belarus and Russia, e. g., from the All-Union Research Institute of Lupine, founded in 1987 (currently a branch of the V.R. Williams Federal Research Center of Forage Production and Agroecology). Germplasm from the German (Gatersleben), Chinese, Australian (CLIMA), Kenyan (National Gene Bank of Kenya, GBK) and other genebanks was constantly added to VIR's holdings. A significant number of accessions were requested and received by mail from the Polish *Lupinus* Gene Bank (Poznan, Poland), including cultivars, local populations, and wild forms of *L. angustifolius* from the centers of origin. Besides, the requested germplasm samples were provided by botanical gardens of the United Kingdom, France, Germany, etc.

In 1991 and 2001, two international collecting teams were sent to Portugal. Thanks to their efforts, the collection was supplemented with 28 accessions, representing local, wild and dedomesticated forms of *L. angustifolius*. Local varieties were also obtained during an expedition to Brazil.

Composition of the narrow-leaved lupine collection at VIR

At present, the collection comprises 887 accessions from 26 countries (Fig. 2).

The largest germplasm shipments came from Belarus, where narrow-leaved lupine breeding dated back to the 1930s. Cv. 'Rozovy 399', developed by Y.N. Svirsky, entered the collection in 1945. Cv. 'Belorussky 155', bred from a tall white-flowered mutant, was included in the holdings in 1952. Accessions of Belarusian origin are represented by cultivars and breeding material with such valuable traits as earliness, productivity, determinate growth type, nonshattering of pods, absence of alkaloids, disease resistance, etc. Among them, there are many varieties that combine a set of genes significant for breeding, i. e., those determining high protein content, disease resistance, nonshattering of pods, low alkaloid content, etc.

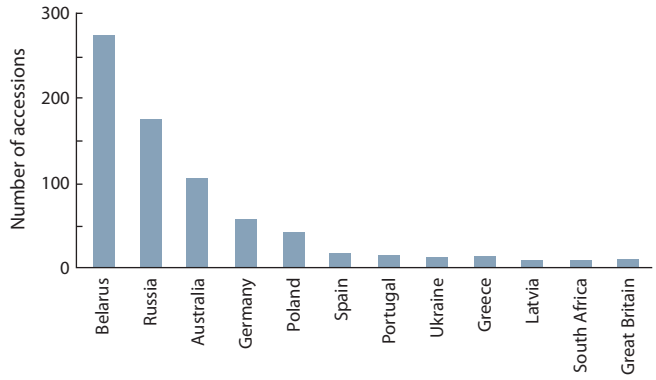

Fig. 2. Numbers of *L. angustifolius* accessions received by VIR from different countries. Only the donor countries of at least 10 accessions are shown.

Intraspecific classifications of narrow-leaved lupine used by VIR

Ecogeographic classification. Researching into the ecotypic structure of the species – a continuation of Vavilov's doctrine of intraspecific differentiation (Vavilov, 1928, 1962) – resulted in an ecogeographic classification based on the analysis of the VIR collection (Kurlovich et al., 1995). Wild forms are classified into geotypes, or ecogeographic groups of ecotypes. Of the 7 identified geotypes, 6 occur in the Mediterranean region (Fig. 3). Within the geotypes, ecotypes are distinguished: roadside, rocky, mountainous, green manure, etc.

This classification was not widely accepted by researchers and plant breeders. Nevertheless, it reflected the patterns of intraspecific variability within the gene pool, and the knowledge of them can optimize the search for certain genes and traits to be used in the development of specialized cultivars. It was shown that sources for breeding small-seeded cultivars with large biomass should be sought in the Iberian Peninsula. There, in mountainous areas, one can also find sources of cold tolerance as well as plant samples resistant to anthracnose and gray spot. Accessions from the Balkan Peninsula and Palestine can serve as sources for the development of early-ripening and large-seeded cultivars (Kurlovich et al., 1995).

Agroecological classification. The cultivated diversity of narrow-leaved lupine is represented by agrogeotypes: Australian, German, Polish, North American, and East European, incorporating different cultivar types (Kurlovich et al., 1995). Cultivar types include cultivar groups of the same utilization trend, with similar biological and agronomic properties.

This classification is based on the history and specificity of plant breeding in different countries, shaped by soil and climate conditions, cultivation techniques, breeding traditions, available source materials, etc. For example, Australian and American cultivars are grown in the autumn/winter season. Therefore, resistance to frosts and returns of cold weather in spring is important for American cultivars. Narrow-leaved lupine cultivars developed in Northern Europe (Gresta et al., 2017), Russia and Belarus should have a short growing season and be adapted to a low sum of mean daily temperatures. Including local varieties and wild accessions from the collection into hybridization makes it possible to transfer frost tolerance or disease resistance to new cultivars (Anokhina et al., 2012). However, European breeders use such material in

Fig. 3. Distribution of *L. angustifolius* geotypes (ecogeographic groups) across the Mediterranean region – the center of origin for this species (from: Kurlovich et al., 1995).

their work to a limited extent. In the 1960s, lupine breeders in Australia initiated their breeding program based on a number of elite cultivars from Europe and the United States ("founder cultivars") and observed the effect known as the "bottleneck" of domestication, so they began to actively involve wild forms in crosses. Over time, the proportion of wild ecotypes in the pedigrees of the developed cultivars increased, while the share of "founder cultivars" reduced. As a result of such practice, new high-yielding cultivars were developed in the early 2000s. Their yield exceeded the yield of those released three decades earlier by 81 %, and in addition they acquired resistance to major pathogens and tolerance to herbicides (Cowling, 2020). It should be mentioned that all countries prioritize the development of disease-resistant cultivars.

Agroecological classification indicates specific traits and properties to be looked for in cultivar types as breeding sources. However, due to the progress in plant breeding and substitution of cultivars over the past 25 years, such typification requires upgrading. For example, it ignores cultivar types that serve as sources of traits for breeding cultivars for food or for fish feed in aquaculture, the rapidly developing trends of narrow-leaved lupine utilization.

Botanical classification (Kurlovich, Stankevich, 1990; Kurlovich, 2002; Kuptsov, Takunov, 2006; Vlasova, 2015) systematizes the intraspecific diversity of *L. angustifolius* according to the color of vegetative and generative organs. The classification identifies varieties of narrow-leaved lupine according to the color of the corolla, jointly with the color and pattern of the seed coat, and its subvarieties according to the color and the presence of anthocyanin on vegetative organs. Determinate and fasciated morphotypes are ranked as forms. Such classification enables crop curators to maintain the authenticity of VIR's accessions in the process of their reproduction; it is used by breeders for crop testing and by geneticists to establish linkages of genes.

One of the most challenging tasks in morphological characterization of narrow-leaved lupine accessions is to describe the habitus, and features of branching and fruit formation. Difficulties are associated with the absence of a well-established terminology, variability of characters under the effect of the environment, and the presence of transitional forms.

Morphophysiological classification according to the growth and branching habit of the stem, proposed by N.S. Kuptsov (Kuptsov, 2001), is currently recognized as the most convenient tool for describing the habitus of narrow-leaved lupine accessions. The degree of branching reduction affects the shaping of morphotypes: wild, quasi-wild, pseudo-wild, corymbose, paniculate, spicate, or palmate. The wild type is characterized by indeterminate stem growth and unlimited branching. In this case, the formation of pods and ripening of seeds do not occur synchronously and, as a rule, are delayed. In other morphotypes, branching is to some extent limited (determined) genetically and blocked by inflorescences. Hybridological methods were used to find out the number of genes and the nature of inheritance of traits in different narrow-leaved lupine forms with reduced branching (Adhikari et al., 2001; Oram, 2002; Kuptsov, Takunov, 2006).

The examples of accessions with limited branching from the VIR collection are as follows: *spicate-type* accessions k-3546, k-3695 (Russia), k-3762 (Germany), k-2955, k-3829, k-3830, k-3832 (Belarus), k-3501, k-3502 (Poland); *corymbose-type* k-3923 (Belarus); *paniculate-type* k-3646, k-3641 (Russia); *palmate-type* k-2979, k-2249 (Russia), etc. Different morphophysiological structure of plants determines their biological properties, such as tolerance to crop densification in a monocrop system, growth rates, flowering and maturation synchrony, stable yield, etc.

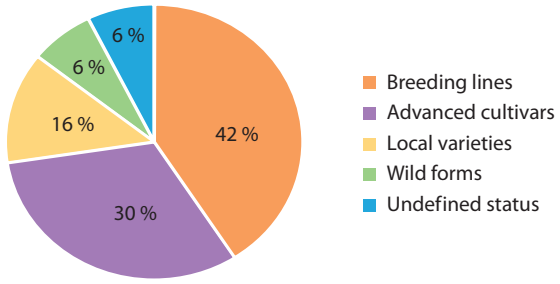

Fig. 4. Pie chart showing the composition of VIR's narrow-leaved lupine collection according to the breeding status of accessions.

Breeding status. The collection of *L. angustifolius* held by VIR includes cultivars developed by scientific breeding (261 accessions), breeding material (370), local varieties (142), wild forms (55), and accessions with an undefined status (50). The percentage of these groups is shown in Fig. 4.

The results of screening VIR's narrow-leaved lupine collection for traits of breeding value

All in all, 640 accessions (73 % of the collection) were assessed for the content of alkaloids in seeds: >1 % –140 accessions (high alkaloid content); 0.4–1.0 % – 23 accessions; 0.1–0.399 % – 50 accessions; 0.025–0.099 % – 230 accessions (low alkaloid content); and <0.025 % – 197 accessions (alkaloid-free). The data were obtained as a result of a rapid field assessment using Dragendorff's reagent (Ermakov et al., 1987) and from published sources. Most of the analyzed accessions (67 %) are low-alkaloid or alkaloid-free. VIR has already tested and selected mass-screening techniques to measure the collection's alkaloid content through chromatographic methods of analysis, thus making such assessments more accurate (Kushnareva et al., 2020).

The collection was studied for resistance to low temperatures. Sources of cold tolerance were identified (Barashkova et al., 1978).

Biochemical screening for the content of protein and oil in seeds revealed the range of variability of these characters and identified accessions with the highest levels of protein (37.9–39.2 %) and oil (7.5–8.4 %) (Benken et al., 1993).

For quite a long time (1971–1987), the collection was being assessed for resistance to *Fusarium* under severe infection pressure, including 2–3 infectious environments created artificially by different methods and located in different regions (Bryansk, Kiev, and Leningrad). Accessions with a very high degree of resistance to the disease were identified, including k-2166, k-2167 (Poland), k-1908, k-2266 (Russia), and k-74 (Belarus). Most of narrow-leaved lupine accessions were

Table 1. Values of main seed yield characters in narrow-leaved lupine accessions from VIR, assessed in Stupino (near Moscow) and Pushkin (near St. Petersburg)

Character	Site	Mean	Min	Max	SD
Plant height (main stem), cm	Stupino District	44.7	33.0	64.8	5.2
	Pushkin	86.6	55.5	123.8	18.21
Number of productive branches	Stupino District	2.6	0.7	5.4	1.2
	Pushkin	5.6	2.8	10.8	1.59
Number of pods per plant	Stupino District	9.2	7.6	26.3	1.1
	Pushkin	35.5	16.2	76.9	13.36
Number of pods on lateral branches	Stupino District	5.0	0.8	15.2	2.9
	Pushkin	28.7	7.3	68.7	13.58
Seed weight per plant, g	Stupino District	4.9	3.3	9.9	1.2
	Pushkin	15.7	3.5	35.0	6.41
1000 seed weight	Stupino District	117.2	67.3	167.5	18.7
	Pushkin	151.1	114.0	186.1	1.85

tested to identify their maturity group and 1000 seed weight (Kiselev et al., 1981, 1988, 1993; Kurlovich et al., 1990).

For many years, VIR's collection of narrow-leaved lupine has been studied at two sites: Mikhnevo Settlement, Stupino District, not far from Moscow, in the Central Non-Black Earth Region of Russia, and the town of Pushkin near St. Petersburg in the Northwestern Region of Russia. The climate in Stupino District is temperate continental, the mean sum of annual active temperatures for many years is 2000–2200 °C, the precipitation amount is 379 mm, and the growing season lasts 130–135 days. The climate in Pushkin is temperate and humid, transitional from oceanic to continental, the mean sum of annual active temperatures for many years is 1879 °C, the precipitation amount is 637 mm, and the growing season is 105–125 days. It should be mentioned that under the conditions of Pushkin not all narrow-leaved lupine accessions had enough time to develop mature seeds.

Field phenotyping includes the assessment of main agronomic traits: yield, maturity group, susceptibility to diseases, and yield components, such as branching, number of pods per plant, seed weight per plant, and 1000 seed weight.

In 2009– 2019, accessions with limited branching (a gene pool generated from natural and induced mutations) were studied in Stupino. In Pushkin, narrow-leaved lupine accessions of various origin and status were examined for many years. Comparison of the results showed a smaller range of variation in productivity traits in lupine forms with determinate branching, represented by modern cultivars and breeding material from Russia, Belarus, and European countries (Germany, Poland, Latvia, etc.), versus the very heterogeneous material studied in Pushkin. A lower but stable yield, limited plant height, and the number of branches make such mutants suitable for growing in dense stands, when it is easier to control weeds. They do not require defoliation to accelerate their maturation, and, on the whole, their harvestability increases (Table 1).

Comparing traits of breeding value in the accessions with limited branching to those in the varieties with indeterminate growth (wild morphotype) under the conditions of Stupino District testified to a lower seed yield in the former group due to a small number of pods developed on lateral branches. During eight years with hot summers, accessions with different branching patterns had similar growing seasons: 60–80 days. But over the course of four years, with summer temperatures dropping to mean values for many years, indeterminate narrow-leaved lupine forms had difficulties with seed development, unless defoliants were applied, or with afterripening, in contrast to determinate ones. Thus, spicate-type accessions have advantages when cultivated in the northern and northwestern regions of the country due to their earliness and synchronous seed maturation.

For the Central Non-Black Earth Region, the main area of narrow-leaved lupine production, a complex of herbivores afflicting lupine crops was identified. The most harmful pests and diseases were *Cerathophorum setosum* Kirchn., *Thielaviopsis basicola* (Berk. & Broome) Ferraris, *Fusarium sambucinum* Fuckel, *Alternaria tenuissimma* (Kunze) Wiltshire, *Pythium mamillatum* Meurs, and *Cylindrocladium* spp. (Golovin, Vlasova, 2015).

In the Russian Northwest, there were massive infestations of lupine aphids (*Macrosiphum albifrons* Essig), symptoms of viral diseases caused by *Phaseolus virus 2* Smith (BYMV, *Bean yellow mosaic virus*) and *Cucumis virus 1* Smith (CMV, *Cucumber mosaic virus*). Among the pathogenic fungi, a dominance of representatives of the genera *Fusarium* Link, *Botrytis* P. Micheli ex Pers., *Sclerotinia* Fuckel and *Stemphylium* Wallr was observed; saprotrophic fungi from the genera *Alternaria* Nees, *Cladosporium* Link and *Epicoccum* Link were also found. Anthracnose incidence, caused by *Colletotrichum gloesporioides* (Penz.) Penz. & Sacc., was insignificant in both regions.

Comparative analysis of the world's narrow-leaved lupine GR collections according to a set of parameters

Collection size. The world's largest collection of narrow-leaved lupine (2,165 accessions) belongs to Australia (CLIMA). The collection of VIR is the second (887 accessions), followed in decreasing order by the collections of the Scientific and Practical Center of the National Academy of Sciences of Belarus (690), Spain (542), Belarusian State University (371), Poland (361), Portugal (291), Germany (279), and the United States (190). The numbers are given according to the European Central *Lupinus* DB, the authors of this publication (VIR's collection), and Privalov et al. (2020) (Belarusian collections).

Status of accessions. Analysis of the breeding status of 3,894 narrow-leaved lupine accessions presented in the European Central *Lupinus* DB suggests that comparing our collection with the world's collections according to this criterion is problematic. First of all, not all categories of "the status" are understood in the same way. For example, in the European DB there are very few local varieties (1.8 %), while in the VIR collection 16 % are recognized as such. Secondly, the category "weedy" with reference to narrow-leaved lupine appears only in the databases of the Spanish and Portuguese GR collections of *L. angustifolius*. There is no such category in the collection of VIR. In some genebanks, the genus *Lupinus* is represented without any species differentiation. A number of databases do not identify the status of accessions. Mapping populations are present only in the Australian collection, and mutants only in the Australian and Polish collections. That is why any differentiation of the world's narrow-leaved lupine GR according to the status of their accessions is to a certain extent arbitrary. One should recognize as the absolute fact that wild forms prevail over other narrow-leaved lupine accessions in the world's GR collections. For example, they account for 82 % in the collection of the PGR Center in Spain and for 60 % in the national collection of Australia (CLIMA).

The results of our analysis demonstrate that on average the global gene pool of narrow-leaved lupine harbors 62 % of wild forms, 12 % of breeding material (lines, hybrids, mapping populations, etc.), 11 % of cultivars developed by scientific breeding, 11 % of accessions with an undefined status, 2 % of mutants, 1.7 % of local varieties, and 0.7 % of weedy accessions.

The analysis of an older but more representative source that included 5,684 narrow-leaved lupine accessions from 17 collections of the world (Buirchell, Cowling, 1998) also showed the predominance of wild forms and local varieties in those collections. At that time, there were 70 % of them in the Australian collection, 100 % in the collection of Portugal, from 80 to 100 % in three Spanish collections, 78 % in the German collection (Braunschweig), and about 50 % in Poland.

The collection of narrow-leaved lupine at VIR presently consists of the following proportions: 42 % of breeding material, 30 % of cultivars developed by scientific breeding, 16 % of local varieties, 6 % of wild forms, and 6 % of accessions with an undefined status. It means that VIR's narrow-leaved lupine collection possesses significantly more germplasm partially modified by plant breeding (breeders' cultivars and breeding material) than all the world's collections of the species, although the percentage of wild forms is incomparably smaller than in other collections. Meanwhile, the most productive Australian alkaloid-free cultivars were developed by crossing cultivated and wild genotypes, despite the fact that their collection contains elite cultivars from other countries. Australian experts believe that wild forms are also fundamentally important for future improvement of narrow-leaved lupine (Gladstones et al., 1998; Mousavi-Derazmahalleh et al., 2018a; Cowling, 2020).

Phenotypic data. Comparison of these data would be possible only if we refer to a few traits assessed both in the accessions from VIR's collection and those from the Australian genebank (CLIMA). This comparative analysis shows a greater range of variability in these traits in Australian accessions (Table 2). It can be explained by the predominance of wild forms, recombinant inbred lines, mutant and hybrid populations in the Australian holdings, as their aggregate genetic diversity is much wider than that in VIR's collection, where cultivars of scientific breeding and breeding material prevail.

Table 2. Values of some phenotypic characters in the narrow-leaved lupine collections held by VIR and CLIMA (Buirchell, Cowling, 1998)

Character	Genebank	Min	Mean	Max	SD
Plant height, cm	VIR	47.1	70.1	135.1	20.3
	CLIMA	10.0	85.0	170.0	22.3
100 seed weight, g	VIR	7.5	14.3	21.1	–
	CLIMA	2.9	11.3	24.4	3.9
Number of pods of the main stem, pcs	VIR	2.4	6.8	11.3	2.30
	CLIMA	1.0	10.0	27.0	7.7
Protein in seeds, %	VIR	18.0	–	39.2	–
	CLIMA	18.8	31.5	40.6	–

Note. VIR's assessment data: town of Pushkin (59° N, 30° E); CLIMA's data: town of Perth (31° S, 115° E).

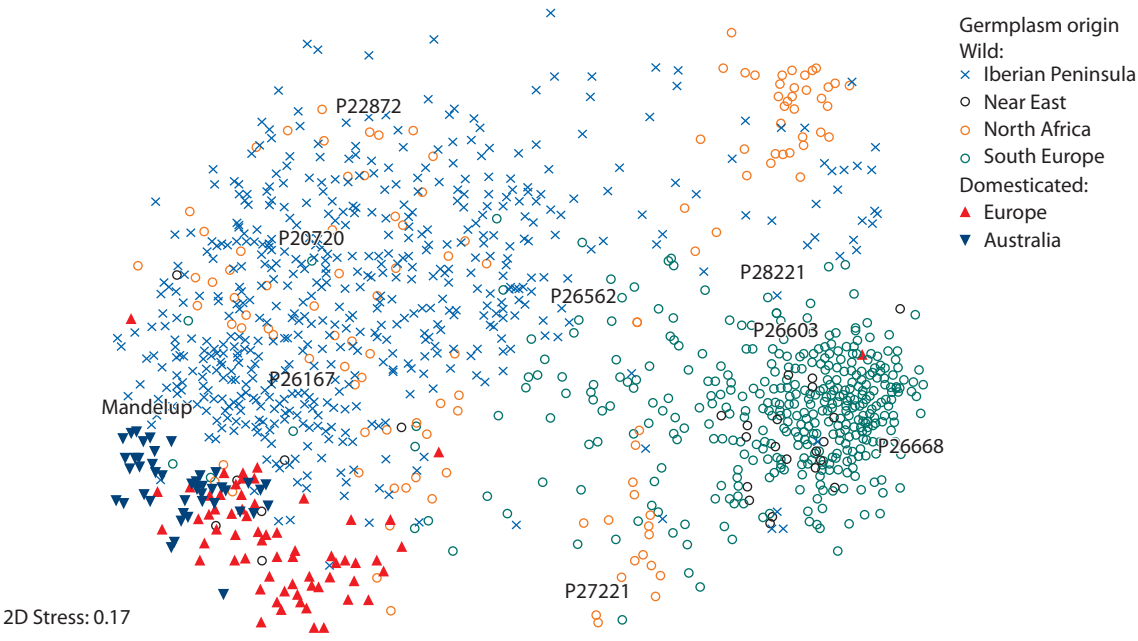

Fig. 5. A multidimensional space scatter plot for *L. angustifolius* accessions from the Australian collection, based on the data from 137 DArT markers (Berger et al., 2012a) classifying accessions according to their domestication status and origin.

The designated accessions (P20720, P22872, P26167, P26562, P26603, P26668, P27221, and P28221) were used in enriching BC_2 crosses with cv. 'Mandelup' (from Berger et al., 2013).

Genetic diversity of wild and cultivated narrow-leaved lupines, identified in different collections

As in most cultivated plants, there is less genetic diversity in domesticated narrow-leaved lupine forms than in wild populations and local varieties, while breeders deal with only a small part of this diversity (Berger et al., 2012a, b). The narrow genetic base of modern cultivars, compared to wild plant forms collected in Southern Portugal, was proved using AFLP and ISSR markers for the analysis of accessions from the Portuguese genebank (Talhinhas et al., 2006). Genotyping the Australian collection of *L. angustifolius* with 137 DArT (Diversity Array Technology) markers showed clear differences between the wild gene pool, collected in various parts of the Mediterranean region, and modern cultivars. At the same time, a difference was revealed between the cultivars developed in Europe and those from Australia, with the presence of a group of cultivars with "overlapping" traits and properties (Berger et al., 2013) (Fig. 5).

Whole-genome sequencing of 146 wild and 85 cultivated accessions from different genebanks of the world ascertained that the genome diversity in modern cultivars is thrice less than in wild populations (Mousavi-Derazmahalleh et al., 2018b).

Methods for identifying differentiation in the gene pool preserved in genebank collections around the world

Today, molecular genetic methods are the most effective tool to identify the degree of diversity and differentiation in a crop gene pool. The Australian narrow-leaved lupine collection was thoroughly studied. There is detailed information on the habitats of its accessions: latitude, longitude and altitude of the sites where they were collected, together with the data describing local climate and soil conditions, often contrasting in these characteristics. Mass phenotyping of the collection and molecular analysis with DArT markers are being performed. Genetic control of key characters and its molecular mechanisms are being studied (Berger, 2013).

Phenotyping and marker-assisted molecular analysis of accessions from the Portuguese collection of narrow-leaved lupine (Instituto Superior de Agronomia Gene Bank) identified three clearly distinguishable large groups of accessions:

1. Mainly forage accessions (one third of them are breeders' cultivars, while the remaining part is breeding material from Europe), combining domestication traits (white flowers, large seeds, water-permeable seed coat, and nonshattering pods). However, their plant habitus is close to the wild type: they are tall and prolifically branching. Flowers contain little anthocyanin.
2. Mostly wild forms, plus several cultivars and breeding lines with strongly shattering pods, petals abundant in anthocyanin, and water-impermeable seed coat. Late-flowering genotypes predominate.
3. Mostly cultivars and breeding lines with low weight of the main stem, relatively short branches, very large seeds, and very large nonshattering pods.

AFLP and ISSR marker techniques grouped modern cultivars as subclusters within an extensive variety of wild germplasm, pointing to a narrower genetic base for domesticated forms (Talhinkas et al., 2006).

Polymorphism of the narrow-leaved lupine gene pool in the content of alkaloids in seeds was demonstrated by the researchers who studied 329 accessions from the Polish genebank in Wiatrowo (Kamel et al., 2015). The study included

143 wild forms and populations collected in the sites of their natural occurrence, 108 accessions representing breeding material, and 78 cultivars developed by scientific breeding. The content of alkaloids varied from 0.0005 to 2.8752 % of seed dry weight. Alkaloid content was high in wild forms and low in cultivars, while the accessions of other statuses were predominantly low-alkaloid. In the collection of the Belarusian State University, the content of alkaloids was significantly lower in the seeds of the narrow-leaved lupine cultivars that were developed later, when compared with older cultivars (Sauk et al., 2008). This collection consists of various cultivars developed by domestic and foreign breeders as well as plant forms obtained through mutagenesis and hybridization between cultivars or lines of *L. angustifolius*.

Almost as soon as plant explorers started collecting samples of wild narrow-leaved lupine forms in the Mediterranean center of the species' origin, Australian scientists disclosed the crop's ecogeographic differentiation (Cowling, 1986; Clements, Cowling, 1994), which made it possible to understand the morphophysiological (adaptive) properties of its natural gene pool. It is known that strong relief dissection and dissimilarities of soil and climate conditions have resulted in significant biological and landscape diversity in this vast territory. Seasonal precipitation, temperatures, relative humidity, insolation, and wind speed are highly variable within the Mediterranean basin (Hijmans et al., 2005). The efforts of Australians to procure knowledge of the ecotypic differentiation in the gene pool of narrow-leaved lupine, in their essence, may be recognized as a continuation of the work initiated by Nikolai Vavilov, who was the first to draw attention to the fact that "species occupying significant areas often demonstrate sharply different ecogeographic complexes of forms" (Vavilov, 1965, p. 246). Almost a hundred years after Vavilov (Vavilov, 1928, 1962), Australian scientists arrived to a similar conclusion, confirming that the identification of adaptabilities in a large number of genotypes across different ecological niches makes it possible to find the address of their further production as crops under appropriate conditions (Berger et al., 2017).

Studying the diversity of narrow-leaved lupine ecotypes in the Mediterranean region facilitated our understanding of the species' reproductive strategy: earliness, and a reduced demand for vernalization and seed dormancy in areas with low precipitation and with droughts in the end of the season. Under such conditions plants bloom earlier, ripen faster, form larger seeds and less biomass, which increases the harvesting index when plant productivity is reduced. The opposite situation is observed in the environments with higher moisture availability and, at the same time, colder weather. Considering these data, phenology can be regarded as a key attribute for the adaptation of wild populations of a species to various habitats within the boundaries of its natural occurrence, and domesticated forms of the species to cultivation areas around the globe (Taylor et al., 2020). The next step is to identify regions associated with climatic adaptation within the genome, in particular, with earliness (Mousavi-Derazmahalleh et al., 2018a).

Genetic variability and phenotypic plasticity were also observed in the architectonics of the root system, when various soil conditions were simulated for growing wild *L. angustifolius* genotypes (Chen et al., 2011).

Thus, the range of studies, revealing the global genetic diversity of narrow-leaved lupine in order to give it the status of a valuable feed and food crop with good adaptive properties and stable productivity, is quite wide. The prospects for improving the crop, considering its youth, are vast. Key traits that determine its economic importance have been identified. There are tools facilitating the search for the sources of these traits in the worldwide gene pool of the species. It is necessary to combine the efforts of scientists, collection holders and plant breeders to exchange the crop's genetic resources in order to enhance its genetic diversity. This need has been tirelessly voiced by Australian experts, working with the GR of narrow-leaved lupine (Buirchell, Cowling, 1998; Berger, 2013; Cowling, 2020; etc.).

Conclusion

The collection of narrow-leaved lupine GR preserved at VIR is represented by a wide variety of accessions with different statuses. Cultivars developed by scientific breeding and breeding material prevail among them. A special place in this gene pool is occupied by accessions with limited branching, most adapted to cultivation in relatively northern regions. They are early-ripening, demonstrate lower but more stable productivity, and are suitable for cultivation in densified stands, which offers a number of agronomic advantages. Comparison of VIR's collection of *L. angustifolius* germplasm with other national collections in the genebanks of lupine-producing countries shows that there are very few wild forms in it. Meanwhile, the collections of other genebanks are rich in wild genotypes. Australia, where the crop's wild gene pool was actively involved in breeding programs, has impressively succeeded in the use of narrow-leaved lupine GR for the development of high-yielding cultivars.

Specific features of the species' reproductive strategy have so far been established, so it can now be adapted to a wide range of environmental conditions. All this calls for further disclosure and exploitation of narrow-leaved lupine's genetic and ecotypic potential, including its wild forms and local varieties, to promote more intensive breeding efforts and large-scale production of this crop in Russia for feed and food purposes. Introgression of the adaptability traits, found in wild and locally cultivated plant forms, into modern cultivars will help to expand the production area of narrow-leaved lupine. This task requires enhancing breeding, genetic, physiological, biochemical and metabolomic studies of the crop's gene pool as well as developing its genomic resources. Identification of the genome regions associated, *inter alia*, with earliness will immeasurably increase the efficiency of searches for source material promising for Russian breeding in GR collections.

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The interphase period "germination–heading" of 8*x* and 6*x* triticale with different dominant *Vrn* genes

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Abstract. The existing spring forms of wheat-rye amphiploids are characterized by late maturity due to the long duration of the interphase period "germination–heading". The manifestation of this trait is influenced by *Vrn-1* genes. Their dominant alleles also determine the spring type of development. The results of studying the interphase period "germination–heading" of spring octaploid and hexaploid forms of triticale created for use in research and breeding programs under the conditions of forest-steppe of Western Siberia are given in this article. The interphase period of the primary forms 8*xVrnA1*, 8*xVrnB1* and 8*xVrnD1* obtained by artificial doubling of the chromosome number of the wheat-rye hybrids made by pollination of three lines of the soft wheat 'Triple Dirk' – donors of different dominant *Vrn-1* genes – by a winter rye variety 'Korotkostebel'naya 69' was determined under the field conditions in the nursery of octaploid (8*x*) triticale. In the nursery of hexaploid triticale, this trait was studied in the populations of hybrids obtained by hybridization of these three primary forms of octaploid triticale with the hexaploid winter triticale variety 'Sears 57'. In the offspring of crossing 8*xVrnD1* × 'Sears 57', spring genotypes of 6*x* triticale bearing *Vrn-D1* were selected. This fact was determined by PCR. It means that the genetic material from the chromosome of the fifth homeologous group of the D genome of the bread wheat is included in the plant genotypes. This genome is absent in the winter 6*x* triticale 'Sears 57'. The grain content of spikes of the created hexaploid forms of triticale is superiour to that of the maternal octaploid triticale forms. It was shown that plants of the hybrid populations 8*xVrnA1* × 'Sears 57' and 8*xVrnD1* × 'Sears 57' carrying the dominant alleles *Vrn-A1a* and *Vrn-D1a*, respectively, have a shorter duration of the "germination–heading" interphase period than the initial parental forms of primary 8*x* triticale. The short interphase period of "germination–heading" of the 6*x* triticale is a valuable breading trait for the creation of early maturing and productive genotypes of triticale.
Key words: octaploid; hexaploid triticale; interphase period "germinarion–heading"; *Vrn-1* genes; hybrids.

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Межфазный период «всходы–колошение» у 8*x* и 6*x* тритикале с различными доминантными генами *Vrn*

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Аннотация. Существующие коллекционные формы яровых пшенично-ржаных амфиплоидов характеризуются позднеспелостью из-за большой продолжительности межфазного периода «всходы–колошение». На проявление этого признака влияют гены *Vrn-1*, аллели которых в доминантном состоянии обусловливают яровой тип развития. В статье приведены результаты изучения межфазного периода «всходы–колошение» у яровых окта- и гексаплоидных форм тритикале, создаваемых для использования в исследовательских и селекционных программах в условиях лесостепи Западной Сибири. Исследования проводили в питомнике октаплоидных (8*x*) тритикале в полевых условиях у первичных форм 8*xVrnA1*, 8*xVrnD1* и 8*xVrnB1*, полученных искусственным удвоением числа хромосом пшенично-ржаных гибридов от опыления пыльцой озимой ржи (сорт Короткостебельная 69) трех линий мягкой пшеницы Triple Dirk – доноров разных доминантных генов *Vrn-1*. В питомнике гексаплоидных (6*x*) тритикале изучали этот признак растений в популяциях гибридов от скрещиваний трех форм первичных октаплоидных тритикале с гексаплоидным озимым сортом тритикале Сирс 57. С помощью молекулярных маркеров у гибридов определен аллельный состав генов *Vrn-1*. В потомстве, полученном от скрещивания 8*xVrnD1* × Сирс 57, выделены и определены методом ПЦР генотипы яровых растений 6*x* тритикале с доминантным геном *Vrn-D1*. Данный факт свидетельствует о включении в них генетического материала хромосомы пятой гомеологичной

группы генома D мягкой пшеницы, входящего в геномный состав октаплоидного тритикале. Этот геном отсутствует в озимом 6*x* тритикале Сирс 57. У созданных гексаплоидных форм тритикале озерненность колоса была лучше, чем у материнских октаплоидных. Показано, что растения из гибридных популяций 8*xVrnA1*×Сирс 57 и 8*xVrnD1*×Сирс 57, несущие доминантные аллели *Vrn-A1a* и *Vrn-D1a* соответственно, обладают более короткой продолжительностью межфазного периода «всходы–колошение», чем исходные родительские формы первичных 8*x* тритикале. Короткий межфазный период «всходы–колошение» у полученных 6*x* тритикале является селекционно ценным признаком для создания раннеспелых и продуктивных генотипов тритикале.
Ключевые слова: октаплоидные; гексаплоидные тритикале; межфазный период «всходы–колошение»; гены *Vrn-1*; гибриды.

Introduction

Hundreds of winter and spring varieties and collection forms of triticale (×*Triticosecale* Wittmack) or wheat-rye amphiploid (WRA) with genomes of wheat (*Triticum* spp.) and rye (*Secale* spp.) have been made for more than 130-year history of this artificial crop. According to the latest data of the world organization FAO, in 2017, the total area of this crop reached almost 4.17 million hectares and grain production was 15.6 million tons. In the Russian Federation the area of crops decreased up to 171.7 thousand hectares in 2017, compared with the maximum value 274.5 thousand hectares in 2014. Grain yield for those years amounted to 500.7 thousand tons and 654.1 thousand tons, respectively (http://www.fao.org/faostat/ru/#data/QC/visualize). This circumstance is due to a decrease in breeding work and creation of varieties of wheat-rye amphiploids in the Russian Federation in recent years.

Hexaploid (6*x*) forms of triticale (B^uB^uAARR, $2n = 42$) are mainly used for agricultural practice. They are more cytogenetically stable and fertile compared to octaploid (8*x*) ($B^uB^uAADDRR$, $2n = 56$) ones (Lukaszewski, Gustafson, 1987). However, there are some reports of successful cultivation of 8*x* triticale (Cheng, Murata, 2002).

In Siberia, this crop has not yet been cultivated on a large scale, since selection of spring triticale forms has not been conducted. Spring triticale samples from the world collection of VIR are late-maturing and winter varieties of European selection do not have good winter hardiness and often do not give good grain yield under severe climatic conditions of Siberia. Two winter short-stemmed varieties Sears 57 and Cecad 90 have been created in Siberian Research Institute of Plant Production and Breeding – Branch of the Federal Research Center the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (SibRIPP&B – Branch of ICG SB RAS) for grainforage use. They occupy only several thousands hectares. More than a dozen of spring varieties have been created in Russia (Tyslenko et al., 2016), but there are no Siberian varieties of spring triticale yet, although spring crops yield yearly, unlike winter ones. In order to carry out breeding work with spring triticale successfully, it is necessary to comprehensively study the characteristics associated with the productivity and adaptability of plants, including those related to the type and duration of plant development.

The type of plant development (spring, winter, alternative), and the duration of the growing period are controlled by *Vrn* (response to vernalization) genes. The key role in wheat species is played by dominant *Vrn-1* genes: *Vrn-A1*, *Vrn-B1*, and *Vrn-D1* (Yan et al., 2003; Muterko et al., 2015, 2016; Shcherban et al., 2015; Dixon et al., 2019). They are in the long arms of 5A, 5B, and 5D chromosomes of three genomes of soft wheat A, B, and D, respectively. There are also the *Vrn-D4* gene located in the centromere region of 5D chromosome (Yoshida et al., 2010; Kippes et al., 2015) and the *Vrn-B3* gene located in the short arm of chromosome 7B (Yan et al., 2006). Studies have revealed the presence of several alleles in each of the *Vrn-1* genes (Yan et al., 2004; Fu et al., 2005; Shcherban et al., 2012; Muterko et al., 2015). The dominant state of any of these genes leads to the spring type of development, and the recessive state leads to the winter type (Pugsley, 1971; Worland, 1996; Yan et al., 2003, 2004, 2006; Fu et al., 2005). The type of plant development of rye is controlled by the *Vrn-R1* gene located in the long arm of the chromosome 5R (Plaschke et al., 1993).

Vrn-1 genes of spring wheat varieties mainly determine the duration of the phases from tillering to tube formation. Duration of the period from germination to heading depends on the allelic state of *Vrn-1* genes. It was shown that the plants containing *Vrn-B1c* allele formed spikes earlier than those with *Vrn-B1a* allele (Emtseva et al., 2013). The expression of *Vrn-A1a* allele leads to earlier earing than that of *Vrn-B1a* or *Vrn-B1c* allele (Kruchinina et al., 2017). The dominant gene *Vrn-A1* of soft wheat *T. aestivum* L. has the greatest effect and the dominant gene *Vrn-B1* has the smallest one (Košner, Pánková, 2004). In octaploid triticale lines created on the basis of almost isogenic on dominant genes *Vrn-1* lines of soft wheat Triple Dirk, the plants with *Vrn-A1a* and *Vrn-D1a* genes formed spikes earlier than those with the *Vrn-B1a* gene (Stepochkin, Emtseva, 2017).

The purpose of this article is to study the duration of the interphase period "germination–heading" of created in SibRIPP&B – Branch of ICG SB RAS spring octaploid and hexaploid triticale forms having different dominant *Vrn-1* genes under conditions of the forest-steppe of Western Siberia.

Materials and methods

The duration of the interphase period "germination–heading" of octaploid (8*x*) and hexaploid (6*x*) triticales with different dominant *Vrn-1* genes affecting the duration of the vegetation period of plants was studied in generations: F_1 – in 2014, F_3 – in 2016, F_4 – in 2017, F_5 – in 2018 and F_6 – in 2019.

Three primary 8*x* triticale forms were made in SibRIPP&B – Branch of ICG SB RAS by crossing almost isogenic lines of soft wheat Triple Dirk D, Triple Dirk B and Triple Dirk E (Pugsley, 1971, 1972) with winter diploid rye variety Korotkostebel'naya 69 and by subsequently doubling the chromo-

some number in wheat-rye hybrids (Stepochkin, 2009, 2017). The wheat lines are the sources and donors of dominant genes *Vrn-A1*, *Vrn-B1* and *Vrn-D1*, respectively. The allelic compositions of *Vrn-1* genes of three 8*x* WRA are *Vrn-A1a*, *vrn-B1*, *vrn-D1*, *vrn-R1* (8*xVrnA1*); *vrn-A1*, *Vrn-B1a*, *vrn-D1*, *vrn-R1* (8*xVrnB1*); *vrn-A1*, *vrn-B1*, *Vrn-D1a*, *vrn-R1* (8*xVrnD1*).

Spring hexaploid forms of triticale were made by selecting early-maturing plants in the offspring of F_3–F_4 hybrids between primary 8*x* WRA and winter 6*x* triticale Sears 57 carrying recessive genes *vrn-A1*, *vrn-B1*, *vrn-R1* (Fig. 1). The allelic composition of *Vrn-1* genes in plants of hybrid populations and parent forms was determined by PCR using allele-specific primers. The structure of primers to *Vrn-1* genes and the conditions of PCR are described in articles (Potokina et al., 2012; Likhenko et al., 2015).

Genomic DNA was isolated according to the previously described method (Likhenko et al., 2015). PCR was performed on a BIO-RAD T-100 Thermal Cycler (USA) amplificator in a total reaction mixture of 20 µl, including DNA (50–100 ng/µl) – 1 µl, 10× buffer for Taq polymerase (650 mM Tris-HCl (pH 8.9); 160 mM $(NH_4)_2SO_4$; 25 mM $MgCl_2$; 0.5 % Tween 20) – 2 µl, dNTPs – 2 µl, direct and reverse primer – 0.5 µl each, Taq polymerase (1 unit/µl) – 1 µl, H_2O – up to the final volume of 20 µl. The separation of PCR products was performed by electrophoresis in 1 % agarose gel with the addition of ethidium bromide.

Sowing in the field soil was carried out by hand in the third decade of May (May 21–24 in different years, depending on the weather) in rows of 0.8 m long, 50 seeds in a row on the isolated from other grain crops experimental plot of SibRIPP&B – Branch of ICG SB RAS, where a three–field crop rotation was maintained: vegetables – fallow soil – triticale. During the growing season, phenological observations and evaluations were carried out. Statistical processing of the results was performed using the Student's *t*-test (Dospekhov, 1985).

Results

The evaluation of plants in populations of primary octaploid WRA showed that the duration of the interphase period "germination–heading" in triticale lines 8*xVrnA1* and 8*xVrnD1* in 2018 and 2019 was shorter than that in 2014, 2016 and 2017 (Table 1). In 2019, triticale 8*xVrnA1* had the shortest "germination–heading" period (52.9 days) among all octaploid WRA, while 8*xVrnB1* had the longest one (72.5 days). This period of the maternal line 8*xVrnD1* lasted 53.8 days. In 2019 hexaploid plants of the hybrid population 8*xVrnA1* × 6*x* Sears 57 in comparison with other hexaploid forms had the shortest duration of this period (47.3 days) and plants of the population 8*xVrnB1* × 6*x* Sears 57 had the longest one (57.8 days). Unlike the maternal forms, this period at 6*x* level was 6 and 14 days shorter. 6*x* plants made by crossing 8*xVrnD1* × Sears 57 had the period of development before earing of 53.4 days and did not significantly differ from the spring octaploid parent. When comparing the data of all years of research, one can note that the selection of the most early-maturing plants in each generation led to a significant reduction of the duration of the period from germination to heading of both hexaploid and maternal octaploid triticale forms except for the parental form 8*xVrnB1*. It did not show significant changes in the duration of this period during all years of research.

Fig. 1. Spikes of spring octaploid triticale plants: 8*xVrnA1* (*1*), 8*xVrnD1* (*2*), 8*xVrnB1* (*3*) and winter hexaploid triticale Sears 57 (*4*).

Ear morphology of hexaploid triticale plants differs from that of the original octaploid forms (Fig. 2). All octaploid triticale lines are awnless, and the hexaploid forms have, like the paternal winter variety Sears 57, small rudiments of the

Table 1. Duration of the interphase period "germination–heading" of hybrid hexaploid and maternal octaploid triticale plants with different dominant *Vrn-1* genes

Hybrids and maternal triticale forms	Duration of the interphase period "germination–heading", days (m ± sem)				
	2014	2016	2017	2018	2019
8*xVrnA1* × Sears 57	69.2 ± 1.3*	66.1 ± 3.3	55.0 ± 1.9**	45.1 ± 2.5*	47.3 ± 1.9*
8*xVrnD1* × Sears 57	75.9 ± 2.1**	68.3 ± 1.7	63.3 ± 2.4	55.2 ± 6.7	53.4 ± 1.7
8*xVrnB1* × Sears 57	79.6 ± 4.4	71.7 ± 1.5	68.6 ± 3.4	56.4 ± 2.1**	57.8 ± 1.5**
8*xVrnA1*	65.2 ± 1.7	65.1 ± 2.0	61.7 ± 1.1	51.0 ± 1.8	52.9 ± 1.4
8*xVrnD1*	67.0 ± 1.9	66.3 ± 0.7	63.9 ± 1.0	49.5 ± 2.6	53.8 ± 2.1
8*xVrnB1*	73.6 ± 1.9	71.3 ± 0.9	69.0 ± 3.1	71.2 ± 4.3	72.5 ± 1.6

* $p < 0.05$; ** $p < 0.01$ – significant differences between a hybrid and its parental form of 8*x* triticale.

Fig. 2. Ears of triticale plants: octaploid 8*xVrnA1* (*1*), 8*xVrnB1* (*2*), 8*xVrnD1* (*3*); hexaploid made from crossing 8*xVrnA1* × Sears 57 (*4*), 8*xVrnB1* × Sears 57 (*5*), 8*xVrnD1* × Sears 57 (*6*).

awns, mainly at the end of the spike. The octaploid amphiploid 8*xVrnD1* as well as the hexaploid 6*xVrnD1* derived from it (hybrid 8*xVrnD1* × Sears 57) have hairy spikes – a trait inherited from Triple Dirk E wheat (*VrnD1*).

For practical use, it is important to note that ears of all hexaploid plants are denser and contain more kernels than those of 8*x* triticale (Table 2). In 2019, the number of kernels in ears of 6*x* forms varied from 25.8 grains of hybrids 8*xVrnB1* × Sears 57 up to 36.4 grains of hybrids 8*xVrnA1* × Sears 57, and in octaploid lines – from 9.1 grains in ears of 8*xVrnD1* to 16.4 grains in ears of 8*xVrnA1*. Weight of grains from ear varied in 6*x* forms from 0.76 ± 0.10 to 1.28 ± 0.21 g, and in 8*x* forms – from 0.24 ± 0.03 to 0.50 ± 0.13 g. In addition, grain unit in hexaploid forms is slightly higher than that in 8*x* WRA. No significant differences between hexaploid and octaploid triticale forms were found for the weight of 1000 grains.

The combination of early ripeness, which is largely due to the duration of the interphase period "germination–heading", and the number of grains of ear makes two hexaploid forms 8*xVrnA1* × Sears 57 and 8*xVrnD1* × Sears 57 promising for further breeding work.

The selected early-maturing hexaploid plants were analyzed by PCR using allele-specific primers for *Vrn-1* genes. Their parents – the winter variety triticale Sears 57 and the octaploid maternal forms were taken for comparison (Fig. 3). The analysis showed that the winter variety Sears 57 carries recessive alleles *vrn-A1*, *vrn-B1* and *vrn-D1*. The maternal forms had the following allelic composition: 8*xVrnA1* – *Vrn-A1a*, *vrn-B1*, *vrn-D1*; 8*xVrnB1* – *vrn-A1*, *Vrn-B1a*, *vrn-D1*; 8*xVrnD1* – *vrn-A1*, *vrn-B1*, *Vrn-D1a*. The offspring from the 8*xVrnA1* × Sears 57 cross was heterozygous on the *Vrn-A1* gene because they contained two alleles – *Vrn-A1a* and *vrn-A1*. In addition, a recessive allele *vrn-B1* was revealed, and the alleles of the *Vrn-D1* gene were not determined due to the lack of an amplification product. Plants of the hybrid population 8*xVrnB1* × Sears 57 have a recessive allele *vrn-A1*, and the *Vrn-D1* gene was not amplified in them. As for the *Vrn-B1* gene, the plants are heterozygous and have two alleles *Vrn-B1a* and *vrn-B1*. Two recessive alleles – *vrn-A1*, *vrn-B1*, and one dominant allele – *Vrn-D1a* – were identified in plants obtained from crossing 8*xVrnD1* × Sears 57.

Discussion

Secondary spring 6*x* triticale breeding samples possess dominant alleles of *Vrn-1* genes and were made by hybridization of primary 8*x* WRA carrying dominant alleles of *Vrn-1* genes with a winter 6*x* WRA carrying recessive alleles. At the 6*x* level in the offspring of hybrids in all the studied generations, their *Vrn-1* genes retain almost the same ranking (8*xVrnA1* × 6*x* Sears 57 > 8*xVrnD1* × 6*x* Sears 57 > 8*xVrnB1* × 6*x* Sears 57) as at the 8*x* level in triticale (8*xVrnA1* ≥ 8*xVrnD1* > 8*xVrnB1*) in terms of the effect on the reduction of the "germination–heading" period. The original Triple Dirk lines have the same ranking of these genes (Stepochkin, 2009; Stepochkin, Emtseva, 2017). Thus, the effect of the dominant alleles *Vrn-A1a* and *Vrn-D1a* leads to a shorter interphase period compared to the effect of the *Vrn-B1a* allele. It is known that in addition to the *Vrn-D1* gene, the *Vrn-D4* gene localized in the chromosome 5D can significantly affect the duration of the period from germination to earing (Kippes et al., 2014). Theoretically, it is possible that along with the *Vrn-D1* gene, a *Vrn-D4* gene can be inserted. However, we exclude this pos-

Table 2. Some ear quantitative characteristics of 8*x* and 6*x* triticale with different *Vrn-1* genes, 2019

Triticale names	Ear density, spikelet number/1 cm	Ear kernel number	Ear kernel weight, g	Weight of 1000 kernels, g	Grain unit, g/1000 cm^3
8*xVrnA1*	2.01 ± 0.06*	16.4 ± 3.1**	0.50 ± 0.13*	30.5 ± 4.0	563 ± 16*
8*xVrnB1*	1.89 ± 0.07**	10.2 ± 2.7**	0.25 ± 0.04**	25.4 ± 4.0	538 ± 2*
8*xVrnD1*	1.95 ± 0.06**	9.1 ± 1.4**	0.24 ± 0.03**	26.7 ± 2.0	578 ± 13
8*xVrnA1* × Sears 57	2.52 ± 0.08	36.4 ± 4.4	1.28 ± 0.21	35.1 ± 2.5	650 ± 10
8*xVrnB1* × Sears 57	2.60 ± 0.04	25.8 ± 2.2	0.76 ± 0.10	29.2 ± 2.1	591 ± 8
8*xVrnD1* × Sears 57	2.74 ± 0.07	36.1 ± 3.1	1.20 ± 0.10	33.1 ± 1.4	589 ± 12
Sears 57 (6*x*)	2.87 ± 0.04	30.0 ± 1.9	0.92 ± 0.17	30.8 ± 6.1	542 ± 9

* $p < 0.05$; ** $p < 0.01$ – significant differences between a hybrid and its parental form of 8*x* triticale.

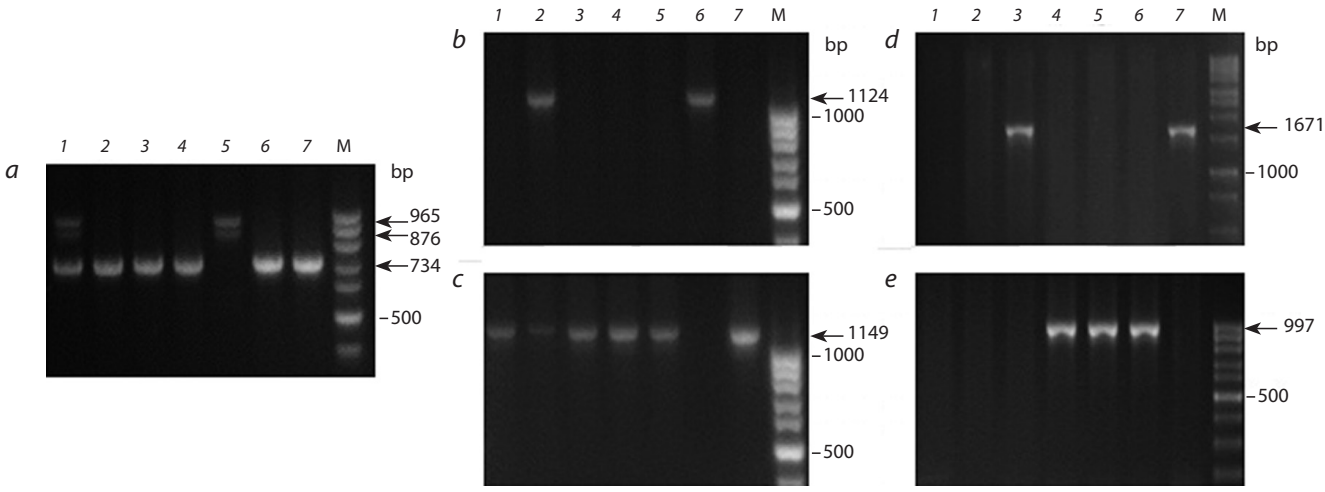

Fig. 3. Amplification of the PCR products using primers for *Vrn-1* genes in hexaploid hybrids of triticale and parental forms: (*a*) *Vrn-A1a* (965 + 876 bp) and *vrn-A1* (734 bp); (*b*) *Vrn-B1a* (1124 bp); (*c*) *vrn-B1* (1149 bp); (*d*) *Vrn-D1a* (1671 bp); (*e*) *vrn-D1* (997 bp).

1–3 – hybrids: 8*xVrnA1* × Sears 57, 8*xVrnB1* × Sears 57 and 8*xVrnD1* × Sears 57, respectively; *4* – winter variety Sears 57; *5–7* – spring octaploid forms: 8*xVrnA1*, 8*xVrnB1* and 8*xVrnD1*, respectively; M – marker of the length of DNA fragments (*a–c*, *e* – 100 bp ladder; *d* – 1000 bp ladder).

sibility, because to make primary octaploid triticales, we used the *Vrn*-isogenic wheat lines Triple Dirk D, Triple Dirk B, and Triple Dirk E, carrying, respectively, only the *Vrn-A1*, *Vrn-B1*, and *Vrn-D1* genes. A comparison of a set of 8*x* triticale lines and 6*x* samples from the VIR world collection showed that the interphase period "germination–heading" of hexaploid triticales is shorter (Stepochkin, Emtseva, 2017). There is an assumption that reducing the level of ploidy can reduce the duration of the period "from germination to heading" in wheat-rye amphiploids. In particular, it was reported that within the crossing combination, octaploid lines formed ears later than hexaploid ones (Kaminskaya et al., 2005).

The hexaploid paternal variety Sears 57 (genomic formula B^uB^uAARR), has a winter type of development. All its *vrn-1* genes have recessive alleles. The maternal forms are three spring octaploid triticale lines (genomic formula $B^uB^uAADDRR$). Each of them carries one dominant gene: 8*xVrnA1* carries a *VrnA1a* allele on the chromosome 5A, 8*xVrnB1* contains a *VrnB1a* allele on the chromosome 5B, 8*xVrnD1* has a *Vrn-D1a* allele on the 5th chromosome of D genome. It was assumed that in 8*xVrnD1* × Sears 57 hybrids in subsequent generations, starting from F_2, the chromosomes of the haploid D genome would be lost during the process of meiosis, and the share of winter plants in the hybrid populations would increase. As a result, in the older generations there would be only winter hexaploid forms with the chromosome number 42 without the haploid genome D and without the dominant allele *Vrn-D1a*. The facts of complete elimination of chromosomes of D genome in such types of crossing are known (Hao et al., 2013). However, by selecting spring plants we were able to create up to the fourth generation populations of 6*x* forms that could begin transition to the generative development after spring sowing without vernalization. Molecular genetic analysis using the PCR method showed the presence of the dominant *Vrn-D1a* allele in these forms (see Fig. 3). This means that either as a result of chromosome substitution or translocation, the *Vrn-D1* gene remained in the complex genome of hexaploid plants. Some researchers report inclusion of a genetic material of the wheat genome D in the genome of hexaploid triticale forms (Kaminskaya et al., 2005). Unlike plants of 8*xVrnD1* × Sears 57 population, hexaploid triticale forms made by crosses 8*xVrnA1* × Sears 57 and 8*xVrnB1* × Sears 57 do not contain any *Vrn-D1* allele, as it was shown by molecular analysis with primers to *Vrn-D1* gene, although the maternal lines contain a recessive *vrn-D1* allele. The lack of amplification is probably due to the elimination of chromosomes of the D genome.

It is known that octaploid triticales are cytogenetically unstable. As a result of disturbances in meiosis, gametes with an unbalanced number of chromosomes are formed, which leads to appearance of aneuploid plants in 8*x* WRA populations (Vettel, 1960a, b; Krolow, 1962, 1963). Hexaploid triticale plants with dominant *Vrn-1* genes may arise as a result of spontaneous depoliploidization of octaploid WRA carrying these genes. This process is accompanied by the predominant elimination of the chromosomes of D genome of soft wheat in octaploid WRA. At the end of this process, stable 6*x* triticales appear, which was found in populations of a number of 8*x* triticales (Stepochkin, 1978; Li et al., 2015).

Conclusion

The presented results showed that the populations of spring octaploid triticales made and maintained at SibRIPP&B – Branch of ICG SB RAS are donors of different dominant *Vrn-1* genes. These populations are used to produce new forms of 8*x* and 6*x* WRA and for breeding process. In the hexaploid triticale forms made on their basis, the allelic composition of the *Vrn-1* genes was determined using molecular genetic analysis. It was found that plants from the populations of 8*xVrnA1* × Sears 57 and 8*xVrnB1* × Sears 57 have genes *Vrn-A1* and *Vrn-B1* in a heterozygous state, so it is necessary to conduct further selection to make homozygous genotypes. In the

created hexaploid forms of triticale, the grain number from ear is higher than that in the original octaploid lines. It is shown that the plants from the hybrid populations 8*xVrnA1* × Sears 57 and 8*xVrnD1* × Sears 57, carrying the dominant alleles *Vrn-A1a* and *Vrn-D1a*, respectively, have a shorter duration of the interphase period "germination–heading" than the original parent forms of the primary 8*x* triticale, which is a breeding-valuable feature for the creation of early-maturing and productive genotypes of triticale.

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Wild grasses as the reservoirs of infection of rust species for winter soft wheat in the Northern Caucasus

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Abstract. Common winter wheat is the main grain crop cultivated in the North Caucasus. Rust disease damage is one of the factors limiting wheat productivity. There are three species of rust in the region: leaf (*Puccinia triticina*), stem (*P. graminis*) and stripe rust (*P. striiformis*), and their significance varies from year to year. The most common is leaf rust, but in the last decade the frequency of its epiphytotic development has significantly decreased. At the same time, an increase in the harmfulness of stripe rust (*P. striiformis*) is noted. Stem rust in the region is mainly absent or observed at the end of the wheat growing season to a weak degree. Only in some years with favorable weather conditions its mass development is noted on susceptible cultivars. It is believed that the sources of infection with rust species in the North Caucasus are infested soft wheat crops, wild-growing cereals and exodemic infection carried by air currents from adjacent territories. In the North Caucasus, forage and wild grasses are affected by *Puccinia* species almost every year. Depending on weather conditions, the symptom expression is noted from late September to December and then from late February to May–June. Potentially, an autumn infection on grasses can serve as a source for infection of winter soft wheat cultivars sown in October. The purpose of these studies is to characterize the virulence of *P. triticina*, *P. graminis*, *P. striiformis* on wild cereals and to assess the specialization of causative agents to winter wheat in the North Caucasus. Infectious material represented by leaves with urediniopustules of leaf, stem and stripe rusts was collected from wild cereals (*Poa* spp., *Bromus* spp.) in the Krasnodar Territory in October–November 2019. Uredinium material from *P. triticina*, *P. striiformis*, and *P. graminis* was propagated and cloned. Monopustular *Puccinia* spp. isolates were used for virulence genetics analysis. In experiments to study the specialization of rust species from wild-growing cereals on common wheat, 12 winter cultivars were used (Grom, Tanya, Yuka, Tabor, Bezostaya 100, Yubileynaya 100, Vekha, Vassa, Alekseich, Stan, Gurt, Bagrat). These cultivars are widely cultivated in the North Caucasus region and are characterized by varying degrees of resistance to rust. Additionally, wheat material was inoculated with Krasnodar populations of *P. triticina*, *P. striiformis*, *P. graminis* from common wheat. In the virulence analysis of *P. triticina* on cereal grasses, four phenotypes (races) were identified: MCTKH (30 %), TCTTR (30 %), TNTTR (25 %), MHTKH (15 %), and five were identified in *P. graminis* (RKMTF (60 %), TKTTF, RKLTF, QKLTF, LHLPF (10 % each). Among *P. striiformis* isolates, three phenotypes were identified using the International and European sets of differentiating cultivars – 111E231 (88 %), 111E247 (6 %) and 78E199 (6 %). Using isogenic Avocet lines, 3 races were also identified, which differed among themselves in virulence to the *Yr1*, *Yr11*, *Yr18* genes (with the prevalence of virulent ones (94 %)). Composite urediniums' samples (a mixture of all identified races) of grass rust of each species were used to inoculate winter wheat cultivars. The most common winter wheat cultivars (75 %) were characterized by a resistant response when infected with *P. graminis* populations from common wheat and cereal grasses. All these cultivars were developed using donors of the rye translocation 1BL.1RS, in which the *Lr26*, *Sr31*, and *Yr9* genes are localized. The number of winter wheat cultivars resistant to leaf rust in the seedling phase was lower (58 %). At the same time, all the studied cultivars in the seedling phase were susceptible to *P. striiformis* to varying degrees. The virulence analysis of the leaf, stem and stripe rust populations did not reveal significant differences in the virulence of the pathogens between wild-growing cereals and soft wheat. Urediniomaterial of all studied rust species successfully infested soft wheat cultivars. The results obtained indicate that grasses are rust infection reservoirs for common wheat crops in the North Caucasus.

Key words: *Puccinia triticina*; *P. graminis*; *P. striiformis*; virulence; resistance; *Triticum aestivum*; *Lr*-genes; *Sr*-genes; *Yr*-genes.

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Злаковые травы – резерваторы инфекции видов ржавчины для озимой мягкой пшеницы на Северном Кавказе России

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Аннотация. Озимая мягкая пшеница – основная зерновая культура, возделываемая на Северном Кавказе. Поражение ржавчинными болезнями – один из факторов, лимитирующих урожайность пшеницы. В регионе отмечаются три вида ржавчины: бурая (*Puccinia triticina*), стеблевая (*P. graminis*) и желтая (*P. striiformis*), значимость которых варьирует по годам. Наиболее распространена бурая ржавчина, но в последнее десятилетие частота ее эпифитотийного развития существенно снизилась. При этом возрастает вредоносность желтой ржавчины (*P. striiformis*). Стеблевая ржавчина в регионе преимущественно отсутствует или наблюдается в слабой степени в конце вегетации пшеницы. В отдельные годы с благоприятными погодными условиями наблюдается ее массовое развитие на восприимчивых сортах. Считается, что источниками инфекции видов ржавчины на Северном Кавказе служат зараженные посевы мягкой пшеницы, дикорастущие злаки и экзодемичная инфекция, заносимая воздушными потоками с сопредельных территорий. На Северном Кавказе практически ежегодно встречается поражение кормовых и дикорастущих злаковых трав видами *Puccinia*. В зависимости от погодных условий проявление симптомов отмечается с конца сентября до декабря и затем с конца февраля до мая-июня. Потенциально осенняя инфекция на травах может служить источником для заражения сортов озимой мягкой пшеницы, высеваемых в октябре. Цель настоящих исследований – охарактеризовать вирулентность *P. triticina*, *P. graminis*, *P. striiformis* на диких злаках и оценить их специализацию к озимой мягкой пшенице на Северном Кавказе. Инфекционный материал, представленный листьями с урединиопустулами бурой, стеблевой и желтой ржавчины, был собран в Краснодарском крае на дикорастущих злаках (*Poa* spp., *Bromus* spp.) в октябре-ноябре 2019 г. В лабораторных условиях урединиоматериал *P. triticina*, *P. striiformis* и *P. graminis* был размножен и клонирован. Монопустульные изоляты видов *Puccinia* использовали для анализа вирулентности. В экспериментах по изучению специализации видов ржавчины с дикорастущих злаков на мягкой пшенице задействовали 12 озимых сортов: Гром, Таня, Юка, Табор, Безостая 100, Юбилейная 100, Веха, Васса, Алексеич, Стан, Гурт, Баграт. Эти сорта широко возделываются в Северо-Кавказском регионе и характеризуются разной степенью устойчивости к ржавчинам. Дополнительно материал пшеницы инокулировали краснодарскими популяциями *P. triticina*, *P. striiformis*, *P. graminis* с мягкой пшеницы. В анализе вирулентности *P. triticina* на злаковых травах выявили четыре фенотипа (расы): MCTKH (30 %), TCTTR (30 %), THTTR (25 %), MHTKH (15 %), а *P. graminis* – пять фенотипов: RKMTF (60 %), TKTTF, RKLTF, QKLTF, LHLPF (по 10 %). Среди изолятов *P. striiformis* с применением международного и европейского наборов сортов-дифференциаторов определены три фенотипа: 111E231 (88 %), 111E247 (6 %) и 78E199 (6 %). С использованием изогенных линий Avocet также идентифицированы три расы, которые различались между собой по вирулентности к генам *Yr1*, *Yr11*, *Yr18* (с доминированием вирулентных (94 %)). Сборные урединиообразцы (смесь всех идентифицированных рас) каждого вида ржавчины со злаковых трав были задействованы для инокуляции сортов озимой пшеницы. Большинство сортов озимой мягкой пшеницы (75 %) характеризовались устойчивой реакцией при заражении популяциями *P. graminis* с мягкой пшеницы и злаковых трав. Все эти сорта созданы с участием доноров ржаной транслокации 1BL.1RS, в которой локализованы гены *Lr26*, *Sr31* и *Yr9*. Число сортов озимой пшеницы, устойчивых в фазе проростков к бурой ржавчине, было ниже (58 %). При этом все изученные сорта в фазе проростков в разной степени были восприимчивы к *P. striiformis*. Проведенный анализ вирулентности популяций возбудителей бурой, стеблевой и желтой ржавчины не выявил существенных различий в вирулентности патогена на дикорастущих злаковых травах и мягкой пшенице. Урединиоматериал всех изученных видов ржавчины успешно заражал сорта мягкой пшеницы. Полученные результаты указывают на то, что злаковые травы являются резерваторами инфекции ржавчин для посевов мягкой пшеницы на Северном Кавказе.

Ключевые слова: *Puccinia triticina*; *P. graminis*; *P. striiformis*; вирулентность; устойчивость; *Triticum aestivum*; *Lr*-гены; *Sr*-гены; *Yr*-гены.

Introduction

Common winter wheat is the main grain crop cultivated in the North Caucasus. Its sown area in this region is more than 7 million hectares (ha), including 1.5 million ha in the Krasnodar Territory, 3 million ha – in the Rostov region, 2.5 million ha ones in the Stavropol Territory and other republics. Leaf disease is one of the factors limiting wheat yield.

Leaf rust is the most common wheat disease in the North Caucasus (caused by *Puccinia triticina* Erikss.). In the last decade, the frequency of its epiphytotic development has significantly decreased compared to the period before 2005. This is due to the gradual increase in the genetic diversity of handled cultivars, timely cultivar change and the lack of a leader cultivar in production.

Wild cereal grasses (*Poa* spp., *Bromus* spp.) with rust species infection (Krasnodar Territory, November 2019).

At the same time, an increase in the importance of stripe rust (*P. striiformis* West.) is noted in the region, which is associated with climate change (long warm autumn, mild winters, lack of soil freezing, prolonged cool springs) (Ablova et al., 2012). Stripe rust is more harmful than leaf rust and can reduce the yield by up to 30 % (Sanin, 2012).

Stem rust (*P. graminis* Pers. f. sp. *tritici* Erikss. & E.) is mainly absent in the region or observed at the end of the wheat growing season to a weak degree. Only in some years with favorable weather conditions its mass development was noted on susceptible cultivars. This is due to wide cultivation in the region of winter wheat cultivars with the *Sr*31 gene, which to this day remains highly effective in protecting against stem rust in Russia. In addition, in the breeding process, the growing season duration of modern cultivars is significantly reduced, which contributes to their escape from the disease (Ablova et al., 2012).

It is believed that the sources of rust species infection in the North Caucasus are the infected crops of soft wheat, wild cereals and exodemic infection carried by air currents from adjacent territories. Winter wheat is sown in September-October. Harvesting takes place from the second half of June to the end of July. Accordingly, rust pathogens urediniospores infection on winter wheat can persist from October to June. Forage and wild-growing grasses (*Bromus*, *Poa*, *Festuca*, *Agropyron*, *Elimus*, *Aegilops*, *Hordeum*, *Agrostis* spp.) can serve as additional infection reservators. Transboundary transfer of rust pathogens urediniospores to the territory of the North Caucasus is possible from Turkey, Iraq, and Iran (Sanin, 2012). According to L.K. Anpilogova et al. (1995), in the North Caucasus, in the epiphytotic years, the infection of wheat stripe rust pathogen appears due to its migration from the Transcaucasia territory to Dagestan, Ossetia, Ingushetia, Kabardino-Balkaria, the foothills and the adjacent steppe of Stavropol and Krasnodar regions.

In the North Caucasus, forage and wild-growing cereal grasses are annually affected by *Puccinia* species (see the Figure). Depending on weather conditions, the symptom expression is observed from late September up to December and then from late February up to May-June. Potentially, an autumn infection on grasses can serve as a source for infection of common winter wheat cultivars sown in October.

The purpose of these studies is to characterize the virulence of *P. triticina*, *P. graminis*, *P. striiformis* on wild cereals and to assess the specialization of causative agents to winter wheat in the North Caucasus.

Materials and methods

Infectious material, represented by leaves with urediniopustules of leaf, stem and stripe rust, was collected on wild cereal grasses (*Poa* spp., *Bromus* spp.) in the Krasnodar Territory in October-November 2019 (see the Figure). The analysis used 18 uredinium samples. The infectious material was dominated by *P. triticina* and. *P. striiformis*. *P. graminis* pustules were of limited abundance. A total of 20 monopustular isolates of *P. triticina*, 16 of *P. striiformis*, and 10 of *P. graminis* were studied.

Infectious material reproducing and obtaining monopustular isolates of *Puccinia* sp. The universally susceptible Michigan Amber winter wheat cultivar was used to propagate an inoculum of rust species and obtain monopustular isolates. Under laboratory conditions, the uredinium material of *P. triticina* and *P. graminis* was propagated and cloned using the method of leaf segments placed in a benzimidazole solution (0.004 %) (Mikhailova et al., 1998). The urediniospores of each monopustular isolate were microscopically investigated to confirm the *Puccinia* species and prevent contamination.

Since the viability of *P. striiformis* urediniospores in the herbarium material is short, the populations were "reanimated" on leaf segments (Mikhailova et al., 1998). For this purpose, the leaves of herbs with urediniopustules were cut into pieces of 5–8 cm and placed in Petri dishes, on the bottom of which two slides were placed. The ends of the leaf segments were covered with cotton wool soaked in a solution of benzimidazole (0.004 %), the Petri dishes were closed and placed in a refrigerator (temperature 3–5° C) for 2–4 days. Such a technique made it possible to stimulate the

resumption of pathogen sporulation. Subsequent propagation of the pathogen was carried out on 10–12-day old wheat plants grown in vessels with soil using the microchamber method. For this purpose, the pieces of leaves with urediniopustules were applied to the leaves and fixed with cling film. Vessels with plants were sprayed with water, covered with plastic wrap frames, and placed into a dark chamber at 10° C for 18–20 h. Then, frames and microchambers with infectious material were removed. Plants were transferred to a Versatile Environmental Test Chamber MLR-352H (SANYO Electric Co., Ltd.), where they were incubated in the light (10,000–20,000 lx) at 16° C for 16 h and then in the dark at 10° C for 8 h (70 % humidity). The symptoms expression was observed 12–18 days after infesting.

Merck vacuum pump (Millipore) (220 V/50 Hz) with special nozzles (1 clone/1 nozzle) was used to collect spores of monopustular rust isolates.

Virulence analysis. The virulence analysis of the populations was carried out using 10–12-day old plants from differentiating lines grown in vessels with soil (1 set/1 monopustular isolate). Plants were sprayed with a spore suspension in a specialized liquid NOVEC 7100, covered with a polyethylene frame to produce a humid chamber and kept in the dark at 20–23° C for leaf and stem rust, at 10° C for stripe rust. After 12 h, the polyethylene was removed and special perforated insulators were pulled over the frames to prevent contamination. Sets of differentiating lines infected by *P. triticina* and *P. graminis* were incubated in a light installation at 20–23° C (photoperiod was 16 h/daytime (illumination 10,000–15,000 lx)/8 h/nighttime), those infected by *P. striiformis* – in the climatic chamber according to the parameters described above.

To study the virulence of the leaf rust causative agent isolates, were used the Thatcher (Tc) lines with genes *Lr1*, *Lr2a*, *Lr2c*, *Lr3a*, *Lr3bg*, *Lr3ka*, *Lr9*, *Lr11*, *Lr14a*, *Lr14b*, *Lr15*, *Lr16*, *Lr17a*, *Lr18*, *Lr19*, *Lr20*, *Lr24*, *Lr26* and *Lr30*; to study the virulence of the stem rust causative agent isolates – the Marquis (Mq) lines with genes *Sr5*, *Sr6*, *Sr7b*, *Sr8a*, *Sr9a*, *Sr9b*, *Sr9g*, *Sr9e*, *Sr9d*, *Sr10*, *Sr11*, *Sr17*, *Sr21*, *Sr24*, *Sr30*, *Sr31*, *Sr36*, *Sr38*, *SrTmp* and *SrMcN*.

The analysis of the stripe rust pathogen was carried out using International (Chinese 166 (*Yr1*), Lee *(Yr7*, *Yr*+), Heines Kolben (*Yr2*+*Yr6*), Vilmorin 23 (*Yr3*), Moro (*Yr10*, *YrMor*), Strubes Dickkopf (*YrSD*, *Yr*+), Suwon 92/Omar (*YrSu*, *Yr*+) and European (Hybrid 46 (*Yr4*, *Yr*+), Reichersberg 42 (*Yr7*, *Yr*+), Heines Peko (*Yr6*, *Yr*+), Nord Desprez (*Yr3*, *YrND*, *Yr*+), Compair (*Yr8*, *Yr19*), Carstens V (*Yr32*, *Yr*+), Spaldings Prolific (*YrSP*, *Yr*+), Heines VII (*Yr2*, *Yr*+)) sets of differentiating cultivars, as well as the Avocet (Ac) lines with genes *Yr1*, *Yr5*, *Yr6*, *Yr7*, *Yr8*, *Yr9*, *Yr10*, *Yr11*, *Yr12*, *Yr15*, *Yr17*, *Yr18*, *Yr24*, *Yr26*, *YrSk(27)*, *YrAR*, *YrSp.* Seed material of cultivars and differentiating lines was kindly provided by A.S. Rsaliev (Research Institute of Biological Security Problems, Kazakhstan).

To designate the races of leaf and stem rust, the North American letter abbreviation was used, according to which the lines were combined into groups (4 lines each) (Long, Kolmer, 1989). The set of lines for stem rust included the lines with genes: *Sr5*, *Sr21*, *Sr9e*, *Sr7b* (group 1); *Sr11*, *S6*, *Sr8a*, *Sr9g* (group 2); *Sr36*, *Sr9b*, *Sr30*, *Sr17* (group 3); *Sr9a*, *Sr9d*, *Sr10*, *SrTmp* (group 4); *Sr24*, *Sr31*, *Sr38*, *SrMcN* (group 5) (Skolotneva et al., 2020); for leaf rust – *Lr1*, *Lr2a*, *Lr2c*, *Lr3* (group 1); *Lr9*, *Lr16*, *Lr24*, *Lr26* (group 2); *Lr3ka*, *Lr11*, *Lr17*, *Lr30* (group 3); *Lr2b*, *Lr3bg*, *Lr14a*, *Lr14b* (group 4); *Lr15*, *Lr18*, *Lr19*, *Lr20* (group 5) (Gultyaeva et al., 2020).

Determination of the stripe rust pathogen races was carried out using International and European differential sets. Decimal nomenclature was used for designation. It is based on a binary designation system for infection types (resistant type of reaction (R) is designated as 0, susceptible (S) as 1) and a decimal designation system for each cultivar (the first differentiating cultivar is 2^0, the second one is 2^1, the third one is 2^2, etc.). Due to the fact that two sets of differentiating cultivars, International and European, were used, when naming a race, first the number according to the International set was written, then the number according to the European one with the prefix E (for example, 1E3) (Gultyaeva, Shaydayuk, 2020).

Immunological studies of winter wheat cultivars in laboratory and field conditions. Krasnodar *P. triticina*, *P. graminis* and *P. striiformis* populations originating from cereal grasses and soft wheat were used for inoculation of the twelve cultivars of common winter wheat: Grom, Tanya, Yuka, Tabor, Bezostaya 100, Yubileynaya 100, Vekha, Vassa, Alekseich, Stan, Gurt, Bagrat. These cultivars are widely cultivated in the North Caucasus region and are characterized by varying degrees of resistance to rust.

Under laboratory conditions, the plants were grown in plastic containers (5–8 grains of each cultivar). At the first leaf phase (10–12-day plants), they were sprayed with each rust species spore suspension in NOVEC 7100. The incubation of infected plants was carried out according to the parameters described above.

The reaction type of differentiating lines and wheat cultivars on leaf rust infection was assessed at 8–10 days according to the E.B. Mains and H.S. Jackson scale (1926), on stem rust infection – at 10–12 days according to the scale of E.C. Stakman et al. (1962), on stripe rust infection – at 16–18 days on the scale of G. Gassner and W. Straib (1928). Plants with scores 0, 1, 2 were classified as resistant, with scores 3, 4, X – as susceptible.

To produce artificial infectious backgrounds in the field conditions of the National Grain Center (Krasnodar Territory), the following methods were used: the plants were inoculated by spraying with an aqueous spore suspension with Tween 80 adhesive, and a wet chamber was made using plastic bags (for the leaf and stripe rusts). Plants were infected with stem rust using a syringe. The consumption rate or infectious load was 10 mg/m^2 of spores for the leaf rust pathogens, 20 mg/m^2 for the stem and stripe rusts. For successful infestation of leaf rust, the temperature should be

at least 15° C, infestation of stem rust – 18° C, infestation of stripe rust – 10° C. Plants were infected with stripe rust in the booting phase, with leaf and stem rust – in the booting and then in the earing phase.

Resistance to rust species was determined by qualitative (reaction type) and quantitative indicators (damage intensity). The reaction type to leaf rust was determined on the E.B. Mains and H.S. Jackson scale (1926), to yellow (stripe) rust – on the G. Gassner and W. Straib scale (1928), to stem rust on the scale of E.C. Stakman et al. (1962). The plants' damage was visually determined: leaf rust infection on flag and pre-flag leaves, stripe rust infection on three upper leaves, stem rust infection on two upper internodes, sheaths of flag and pre-flag leaves. The damage intensity by leaf and stem rusts was determined according to the Peterson scale, and by stripe rust – the modified Cobb scale (McIntosh et al., 1995). Damage intensity registration by rust types was carried out in the period from earing up to milky-wax ripeness.

Results and discussion

The urediniospores of *P. triticina*, *P. graminis*, and *P. striiformis* from wild cereals successfully infected the universally susceptible winter wheat cultivar Michigan Amber, which made it possible to carry out population genetic studies of pathogens and immunological studies of wheat cultivars. In the analysis of *P. triticina* virulence, 20 monopustular isolates were studied and four races (phenotypes) were identified: MCTKH (30 %), TCTTR (30 %), TNTTR (25 %), MHTKH (15 %). All isolates were avirulent to the Thatcher lines with genes *Lr9*, *Lr19*, *Lr24* and virulent to *Lr1*, *Lr3a*, *Lr3bg*, *Lr3ka*, *Lr11*, *Lr14a*, *Lr14b*, *Lr17a*, *Lr18*, *Lr20*, *Lr26*, *Lr30*. Frequencies variation was observed on the lines Tc*Lr2a*, Tc*Lr2b*, Tc*Lr2c*, Tc*Lr15* (55 % virulence) and Tc*Lr16* (40 %).

When analyzing the *P. graminis* population, a higher phenotypic diversity was observed. Five races (RKMTF (60 %), TKTTF, RKLTF, QKLTF, LHLPF (10 % each)) were identified among the 10 monopustular isolates studied. All isolates were avirulent to the Marquis lines with genes *Sr9e*, *Sr11*, *Sr24*, *Sr30*, *Sr31* and virulent to genes *Sr5*, *Sr6*, *Sr9a*, *Sr9g*, *Sr10*, *Sr36*, *Sr38*, *SrTmp*, *SrMcN*. Variability in reaction types was observed on the lines *Sr7b*, *Sr8a*, *Sr9b*, *Sr9e*, *Sr9d*, *Sr17*, *Sr21*.

Virulence to stripe rust was studied using 16 monopustular isolates. All isolates were avirulent to the Moro, Nord Desprez, Compair differentiating cultivars and the Avocet lines with genes *Yr5*, *Yr8*, *Yr10*, *Yr12*, *Yr15*, *Yr17*, *Yr24*, *Yr26* and virulent to the cultivars Lee, Heines Kolben, Vilmorin 23, Hybrid 46, Reichersberg 42, Suwon 92/Omar, Heines Peko, Spaldings Prolific, Heines VII as well as to the lines with genes *Yr6*, *Yr10*, *YrSk(27)*, *YrAR*, *YrSp*. Virulence variations were noted for cultivars Chinese 166, Strubes Dickkopf, Carstens V and the lines Ac*Yr1*, Ac*Yr11* and Ac*Yr18*. According to the International and European sets of differentiating cultivars, *P. srtriiformis* isolates were represented by the races 111E231 (88 %), 111E247 (6 %), and 78E199 (6 %). In the virulence analysis using the Avocet lines, three phenotypes were identified, differing from each other in virulence to *Yr1*, *Yr11*, *Yr18* (with the prevalence of virulent ones (94 %)).

Combined populations of each rust species from cereal grasses were used to infect winter wheat cultivars widely cultivated in the region (Table 1). The inoculum included isolates of all races of the pathogen identified in the virulence assay. Additionally, the studied cultivars were infected with Krasnodar populations of *P. triticina*, *P. striiformis*, *P. graminis* from soft wheat. The *P. triticina* population was avirulent to the Thatcher lines with *Lr* genes: 9, 16, 19, 24 and virulent to *Lr* genes: 1, 2a, 2b, 2c, 3a, 3bg, 3ka, 10, 14a, 14b, 15, 17, 18, 20, 26, 30; *P. striiformis* population was avirulent to *Yr* genes: 5, 10, 15, 17, 24, 26 and virulent to *Yr* genes: 1, 3, 4, 6, 7, 8, 9, 18, 32, Sp; *P. graminis* population was avirulent to *Sr* genes: 24, 30, 31, and virulent to *Sr* genes: 5, 6, 7b, 8a, 9a, 9e, 9d, 10, 21, 36, 38, McN, Tmp.

Most cultivars of winter soft wheat (75 %) were characterized by a resistant reaction when infected with *P. graminis* populations from soft wheat and cereal grasses (see Table 1). Many of them have the 1BL.1RS rye translocation, in which the *Lr26*, *Sr31*, and *Yr9* genes are localized. Despite the fact that the efficiency of the *Sr31* gene has been overcome in a number of countries in the world, the virulence to this gene in the North Caucasus region has not yet been identified. At the same time, the rapid range expansion of the races of Ug99 group, virulent to *Sr31*, and their detection in territories close to the North Caucasus of Russia (for example, Iran) presupposes continuous monitoring of this pathogen populations and improving genetic protection (Nazari et al., 2009).

The number of winter wheat cultivars resistant to leaf rust in the seedling phase was lower (58 %). These included cultivars Tabor, Alekseich, Tanya, Gurt, Yuka, Stan, Bagrat. Cultivars Bezostaya 100 and Vekha were moderately resistant (score 2–2$^+$) to the population of the pathogen from common wheat, but susceptible to the population from cereal grasses. The *Lr26* gene of these cultivars has long lost its effectiveness in Russia. At the same time, pyramiding of this gene with other genes is effective (Sibikeev et al., 2011). The cultivars Grom, Yubileynaya 100 and Vassa belonged to the group susceptible to leaf rust.

All studied cultivars showed various degrees of susceptibility to *P. striiformis* populations from cereal grasses and common wheat (scores 2–3, 3, 3–4). This indicates that genes *Yr9* and *Yr18*, which are widely represented in winter wheat cultivars handled in the North Caucasus, are not effective in protecting against stripe rust in the seedling and tillering phases. Accordingly, such cultivars can accumulate aerogenic infection from cereal grasses and under favorable weather conditions contribute to its appearrance and mass development.

These researches revealed a high diversity in the studied isolates of *Puccinia* species. The races ratio analysis of the rust species on cereal grasses and on wheat crops was of

Table 1. Resistance of common wheat cultivars to rusts at the seedling stage

Cultivar	Resistance genes	Reaction type onto *Puccinia* populations samples, score					
		P. graminis		*P. triticina*		*P. striiformis*	
		Common wheat	Cereal grasses	Common wheat	Cereal grasses	Common wheat	Cereal grasses
Grom	*Lr1*	3–4	3–4	3–4	3–4	3	3–4
Tabor		3^-	3–4	2	0–1	3–4	3
Yubileynaya 100	*Lr34/Sr57/Yr18*	3	3–4	3–4	3–4	3	3–4
Vassa	*Lr26/Sr31/Yr9*	1–2	0–1–2	3–4	3	3–4	3–4
Alekseich		1–2	1–2	0–1	0–1	3	3
Tanya	*Lr26/Sr31/Yr9 Lr34/Sr57/Yr18*	1–2	1–2	1	0–1	3	3
Gurt	*Lr1+Lr26/Sr31/Yr9*	1–2	0	0–1	0–2	3–4	3
Yuka		0–1	1–2	2	1–2	3	3–4
Bezostaya100	*Lr26/Sr31/Yr9 Lr34/Sr57/Yr18*	0	0	2	2^+–3^-	2–3	3
Vekha	*Lr10+Lr26/Sr31/Yr9*	1–2	1–2	2–2^+	3–4	3	3
Stan		1–2	1	0	0–1	3	3
Bagrat	*Lr1+Lr10+Lr26/Sr31/Yr9*	0	0	0	0–1	2–3	3^-

considerable interest. The *P. triticina* races identified on cereal grasses in these researches (MCTKH, MHTKH, TCTTR, THTTR) are regularly noted in analyses of North Caucasian and other Russian populations (Kolmer et al., 1915; Gultyaeva et al., 2020). In 2019, the *P. srtriiformis* 111E247 race dominated Krasnodar and Leningrad pathogen populations on soft wheat, and the 78E199 race dominated the Novosibirsk population (Gultyaeva, Shaidayuk, 2020). The *P. graminis* races RKMTF, TKTTF, RKLTF, QKLTF, LHLPF are similar in virulence to those identified on common wheat in samples of North Caucasian and European populations of this pathogen (Skolotneva et al., 2013; Sinyak et al., 2014).

Our obtained results are consistent with the data presented in the literature (Budarina, 1955; Borisenko, 1970; Lesovoy, Tereshchenko, 1972; Krayeva, Matviyenko, 1974; Paichadze, Yaremenko, 1974; Berlyand-Kozhevnikov et al., 1978; Popov, 1979; Hovmøller et al., 2011; Cheng et al., 2016). In the 1969–1972 research of *P. striiformis* uredinium samples collected on a wide set of wild cereals in the North Caucasus, the 20, 31, 19, 9, etc. races were identified, which are also highly specialized to common wheat (Krayeva, Matviyenko, 1974). The analysis of stripe rust inoculum collected from five cereal grass species (creeping wheatgrass, fibrous regneria, siberian wild rye, cock's-foot grass, redtop) in 1973 in the Altai Territory revealed that isolates from siberian wild rye, fibrous regneria and creeping wheatgrass perform virulence to soft wheat (Popov, 1979). Among the specimens of *P. striiformis* collected on wild cereals in 1968–1972 in the vast territory of Georgia, eight revealed races 20, 31, 40, 19, 42A2, 25, 13, 20A2 are also specialized for soft wheat (Paichadze, Yaremenko, 1974).

A high genetic diversity of stripe rust isolates in terms of virulence on wild-growing cereals has also been shown in other countries (Hovmøller et al., 2011; Cheng et al., 2016). This is due to the balanced genetic diversity of hosts and their parasites in natural biocenoses. For example, among the isolates of *P. striiformis* obtained from 11 species of wild-growing cereals in the USA, isolates that are virulent to common wheat (f. sp. *tritici*), barley (f. sp. *hordei*), both of these species, rye, triticale and other cereals were identified.

Similar results were obtained when studying the causative agent of stem rust in 2000–2009 in the Central region of Russia (Skolotneva et al., 2013). A high similarity in the composition of the pathogen populations on soft wheat and wild cereals was determined. Analysis of the long-term dynamics of the main *P. graminis* races showed that the phenotypes that dominate on common wheat and other cultivated cereals in some years do not completely disappear in unfavorable seasons, but remain on wild cereals (Skolotneva et al., 2013).

According to V.M. Berlyand-Kozhevnikov et al. (1978), the main (maternal) population of the leaf rust pathogen in southern Dagestan is a set of pathogen clones that parasitize on wheatgrass and other perennial wild cereals throughout the year. In early spring, and sometimes in autumn, the disease also appears on various annual cereals. The spread of the pathogen population from perennial cereals to wheat crops begins with the development of clones that can para-

Table 2. Immunological characteristic of common wheat cultivars in Krasnodar region under artificial infection conditions (2018–2020)

Cultivar	Rust diseases damage intensity, % and reaction type, score		
	P. graminis	*P. triticina*	*P. striiformis*
Grom	67 S	79 S	30 S
Tabor	60 S	20 MS	40 MS
Yubileynaya 100	80 S	30 MS	60 S
Vassa	10 R	10 MR	30 MS
Alekseich	10 MR	1 R	1 R
Tanya	30 MR	32 MS	30 MS
Gurt	40 MS	60 S	30 MS
Yuka	30 MS	68 S	40 MS
Bezostaya100	10 MR	1 R	10 R
Vekha	10 R	20 MS	20 MR
Bagrat	10 MR	10 MR	20 MR
Stan	10 R	10 MR	40 MS

Note. Reaction type: R – 1 score, MR – 2 scores, MS – heterogeneous reaction type X (2–3), S – 3–4 scores.

sitize on the corresponding host plants. This hypothesis was confirmed by studying the specialization of the leaf rust pathogen in Ukraine forest-steppe conditions in the 1970s (Lesovoy, Tereshchenko, 1972). The isolates of the fungus collected from cereal grasses successfully infested common wheat cultivars and were represented by five races, among which the race 77 dominated. Its frequency in rust samples from *Elitrigia repens*, *Bromus tectorum*, *Festuca pratensis*, *Poa angutifolia* reached 100 %. Other races were found singularly: the race 6 was revealed in *P. trivialis* samples, the race 4 was revealed in *A. imbricatum*, the races 130 and 144 were revealed in *B. mollis*. The fungus population on common wheat had these races in unsignificant numbers. N.A. Budarina (1955) showed that *Ae. cylindrica*, cheat grass (*B. tectotium*) and narrow-ear wheatgrass (*Ag. cristatum* var. *imbricatum*) can serve as leaf rust reservators in the Crimea. Leaf rust infection obtained from these species successfully infected wheat. A.N. Borisenko (1970), when studying *P. triticina* populations on wild cereals in Kazakhstan, Kyrgyzstan, and Western Siberia, identified 10 races on them, and all these races were recorded on common wheat.

Most species of wild-growing cereals are perennials, so rust pathogens can persist in the form of urediniopustules or urediniomycelium for a long period. During the 2019–2020 growing season in the Krasnodar Territory, an air temperature excess over the long-term average values was noted in every month, except April and May. The winter wheat growing season practically did not stop both in autumn and winter.

Despite the fact that in the fall of 2019 in the central and southern foothill zone of the Krasnodar Territory, the foothill zone of the Stavropol Territory, powerful infectious potential of rust pathogens with a dominance of stripe rust was formed on wild and weed cereals, in autumn and winter surveys of breeding and industrial crops of winter wheat no stripe rust symptoms were detected. This is due to insufficiently favorable weather conditions for the wheat infection. Due to the acute limit of moisture supply in spring, low relative humidity, as well as windy weather, the spread and development of phytopathogens on cereal grasses stopped. At the same time, in the 2020 fall, like in 2019, the appearance of rust on wild and forage cereal grasses was noted again, but much less developed than in 2019. This confirms the stability in the preservation of urediniofection of rust species in natural biocenoses as well as the potential contamination of grain crops under favorable weather conditions.

Winter wheat cultivars used in these studies to assess the specialization of rust pathogens are leading in the North Caucasus in terms of sown area. As an example, Tanya cultivar occupies 18 % of the total area under winter wheat in the Krasnodar Territory and has been cultivated in production for more than 15 years; Grom occupies 15 %, Alekseich – 9.5 %, Yuka – 9 % of the winter wheat total sowing area. These cultivars are characterized by different levels of rust resistance when artificially infested in the field. The cultivars Alekseich and Bezostaya 100 have group resistance to three rust species. They have been approved for handling in production since 2017. The Tanya is characterized by high field resistance to *Puccinia* spp. The cultivars Bagrat, Vekha, Vassa, Stan, Tabor, Yubileynaya 100 are classified as moderately resistant to leaf rust. The intensity of their damage varies from 10 up to 40 % with moderately resistant and moderately susceptible reaction types. The Grom is a rust susceptible cultivar. The cultivars with *Sr31* in their haplotype are resistant to stem rust: Vassa, Vekha, Bagrat, Stan – the degree of their damage does not exceed 10 % with resistant and moderately resistant reaction types. In cultivars Gurt and Yuka with similar genetic material, the damage intensity is 30 and 40 %, respectively, with a moderately susceptible reaction type. The Tabor and Yubileynaya 100 show susceptibility to stem rust. The Vekha and Bagrat cultivars have moderate resistance to stripe rust. The average rust incidence on these cultivars at the infectious site of the National Center of Grain during artificial 2018–2020 infection is presented in Table 2.

There were no significant differences in the infection of the studied cultivars by leaf and stripe rust against an artificial infectious background in 2018–2020 compared with the previously presented characteristic (Bespalova et al., 2020). For most cultivars, the results of field assessments correlated with those obtained in the seedling phase.

Immunological studies of winter common wheat cultivars show that despite comparatively similar haplotypes for resistance genes to rust species, the immune activity and its duration are diverse even with the “antimonopoly” law,

which effectively works in the North Caucasus agrophytocenoses. This is due to the growing season duration, the period of "attacking", as well as the infectious load during the critical phases of the host plant ontogenesis for infesting. For stable protection of wheat plants against *Puccinia* spp. it is necessary to use genes that determine various mechanisms of resistance.

Conclusion

The analysis of the racial composition and virulence of the pathogens' populations of leaf, stem and stripe rust indicates that in the conditions of Russian North Caucasus wild cereals are reservators of rust species and, under favorable weather conditions, can serve as a source of infection for common wheat crops and other cultivated cereals. The high diversity of the racial composition of the pathogen in natural cenoses and the wide specialization of *Puccinia* isolates imply the continuous evolution of the pathogen due to the emergence of new mutations in virulence and somatic hybridization, which should be considered in breeding for cereals resistance to rust.

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Variation in nuclear genome size within the *Eisenia nordenskioldi* complex (Lumbricidae, Annelida)

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Abstract. The size of the nuclear genome in eukaryotes is mostly determined by mobile elements and noncoding sequences and may vary within wide limits. It can differ significantly both among higher-order taxa and closely related species within a genus; genome size is known to be uncorrelated with organism complexity (the so-called C-paradox). Less is known about intraspecific variation of this parameter. Typically, genome size is stable within a species, and the known exceptions turn out be cryptic taxa. The *Eisenia nordenskioldi* complex encompasses several closely related earthworm species. They are widely distributed in the Urals, Siberia, and the Russian Far East, as well as adjacent regions. This complex is characterized by significant morphological, chromosomal, ecological, and genetic variation. The aim of our study was to estimate the nuclear genome size in several genetic lineages of the *E. nordenskioldi* complex using flow cytometry. The genome size in different genetic lineages differed strongly, which supports the hypothesis that they are separate species. We found two groups of lineages, with small (250–500 Mbp) and large (2300–3500 Mbp) genomes. Moreover, different populations within one lineage also demonstrated variation in genome size (15–25 %). We compared the obtained data to phylogenetic trees based on transcriptome data. Genome size in ancestral population was more likely to be big. It increased or decreased independently in different lineages, and these processes could be associated with changes in genome size and/or transition to endogeic lifestyle.
Key words: earthworms; *Eisenia nordenskioldi*; genome size; flow cytometry; phylogeny.

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Изменчивость размеров ядерных геномов у представителей комплекса *Eisenia nordenskioldi* (Lumbricidae, Annelida)

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Аннотация. Размеры ядерного генома у большинства эукариот определяются преимущественно содержанием мобильных элементов и некодирующих последовательностей и варьируют в широких пределах. Они могут значительно различаться как между крупными таксонами (причем размер генома не коррелирует со сложностью организма – так называемый С-парадокс), так и между близкородственными видами в пределах рода. В то же время размах внутривидовой изменчивости по этому параметру изучен значительно хуже. Комплекс *Eisenia nordenskioldi* объединяет несколько близкородственных видов дождевых червей, широко распространенных на Урале, в Сибири и на Дальнем Востоке России, заходящих своими ареалами и в сопредельные регионы. Для этого комплекса характерна значительная морфологическая, кариотипическая, экологическая и генетическая изменчивость. Целью настоящей работы было оценить размеры ядерного генома у нескольких филогенетических линий комплекса *E. nordenskioldi* при помощи проточной цитофотометрии. Получены данные о размерах генома для 13 популяций, относящихся к семи филогенетическим линиям *E. nordenskioldi*. Наши результаты показали, что между линиями комплекса наблюдается заметный разброс по размерам, что является еще одним подтверждением их видовой самостоятельности. В целом по размеру генома выборки разделены на две группы. В одну вошли три популяции с небольшим (250–500 м. п. н.), во вторую – с крупным (2300–3500 м. п. н.) размером генома. Кроме того, разные популяции в пределах одной филогенетической линии также имели заметные различия в размере генома (15–25 %). Полученные данные были сопоставлены с филогенетическими деревьями, построенными на основе транскриптомных данных.

Судя по топологии филогенетических деревьев, предковые популяции комплекса с большей вероятностью имели большой размер генома, а уменьшение или увеличение его размера происходило в разных линиях независимо и, возможно, было связано с изменением размеров тела и/или переходом к собственно почвенному образу жизни.
Ключевые слова: дождевые черви; *Eisenia nordenskioldi*; размер генома; проточная цитофотометрия; филогения.

Introduction

The amount of nuclear DNA in eukaryotes varies widely and does not correlate with the complexity of an organism (Cavalier-Smith, 1978; Gregory, 2001). This phenomenon was dubbed the "C-paradox" (Thomas, 1971). Patterns of genome size variation are currently well studied both for higher-level taxa and for groups of closely related species from many diverse phyla (Gregory, 2005). The patterns of intraspecific diversity are generally less known. It is generally believed that genome size and architecture must be common in different populations so they remain genetically and reproductively compatible, i. e. remain a species. There are certain deviations from this rule: differences between males and females due to sex chromosomes; the presence of additional B-chromosomes or large blocks of heterochromatin (Gregory, 2005; Biémont, 2008). However, in most cases intraspecific diversity does not exceed several percent (Blommaert, 2020). The known cases of high variation in intraspecific genome size (Alvarez-Fuster et al., 1991; Marescalchi et al., 1998; Neiman et al., 2011; Stelzer et al., 2011; Jeffery et al., 2016) are often explained by the presence of the so-called cryptic, or sister, species, which were not detected earlier.

The *Eisenia nordenskioldi* (Eisen, 1874) complex is a group of species/genetic lineages of earthworms from the Lumbricidae family widespread in Asian Russia and also found in the East European Plain and certain adjacent countries (Perel, 1979; Zhukov et al., 2007; Blakemore, 2013; Hong, Csuzdi, 2016; Shekhovtsov et al., 2017b). This complex is known for its enormous morphological (Malevich, 1956; Perel, 1979; Vsevolodova-Perel, 1997), karyotypic (Graphodatsky et al., 1982; Vsevolodova-Perel, Bulatova, 2008), ecological (Berman et al., 2019), and genetic (Malinina, Perel, 1984; Shekhovtsov et al., 2013, 2016a, b, 2017a, 2018a, b) diversity. Phylogenetic studies using genomic and transcriptomic data confirmed deep divergence between the lineages of this complex (Shekhovtsov et al., 2019, 2020a, b) and suggested that it could be divided in at least two distinct species.

Remarkable differences between the nuclear and mitochondrial genomes of *E. nordenskioldi* genetic lineages indicate that they diverged long ago (Shekhovtsov et al., 2013, 2015). Significant variation in genome size not associated with polyploidy could thus have accumulated in this complex. To elucidate this question we studied genome size in several genetic lineages of *E. nordenskioldi* using flow cytometry.

Materials and methods

Live earthworms were collected in 2020 in various locations from the Urals, Siberia, and the Far East (see the Table). The warms were rinsed, placed individually in Petri dishes with wet paper and kept for 3–7 days. Genome size was estimated according to the fluorescence of DAPI-stained nuclei of individual cells according to the technique of D.W. Galbraith et al. (1997). Nuclei were isolated either from several posterior segments of a live earthworm (100–300 μg) or from the whole animal if it was small. A part of the material (about 50–100 μg) was fixed in ethanol for DNA extraction as described below.

Live material was placed in a Petri dish with 500 μl of Galbraith buffer: 45 mM $MgCl_2$, 20 mM 3-[N-morpholino] propanesulfonic acid (MOPS), 30 mM sodium citrate, 0.1 % Triton X-100 (Galbraith et al., 1983). Material was grinded by multiple strokes with a razor blade. Liquid phase was transferred into an Eppendorf tube. Another 500 μl of Galbraith buffer was added to the Petri dish, and liquid phase was again transferred to the Eppendorf tube. The sample was incubated for 15–60 min, filtered through a 40 μm mesh, and placed on top of 2 ml Galbraith buffer with 3 % glycerol. The tube was centrifuged for 10 min at 200 g; supernatant was discarded, the sediment was dissolved in 500 μl of Galbraith buffer with 10 μl RNAse (1 u/μl). The sample was incubated for 30 min, mixed with 100 μl of propidium iodide (1 mg/ml) and analyzed on a FACSAria III flow cytometer (BD Biosciences, USA). We used chicken blood cells (2C = 1250 Mbp) (Kasai et al., 2012) and mouse spleen cells (2C = 3280 Mbp) (Redi et al., 2005) as the reference.

To determine genetic lineage, we sequenced a fragment of the mitochondrial cytochrome oxidase I gene as described in (Shekhovtsov et al., 2018c). Phylogenetic trees built using the Maximum Likelihood and Bayesian inference algorithms were taken from S.V. Shekhovtsov et al. (2020b).

Results and discussion

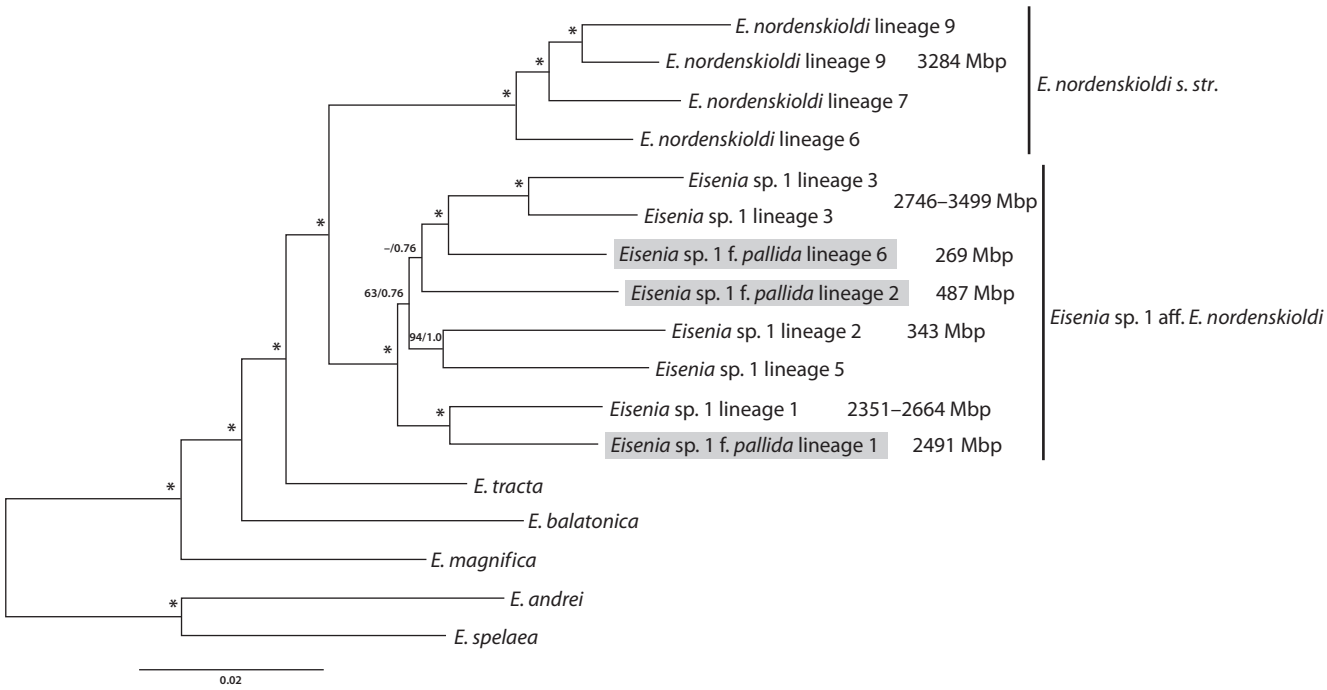
In this study we determined genome size for several genetic lineages of the *E. nordenskioldi* complex (see the Table and Figure). The obtained data indicate high variation in genome size in this complex. We could distinguish two size classes: small (250–500 Mbp) and large (2350–3500 Mbp) genomes. Small genomes were observed in three cases, for two non-pigmented lineages of *Eisenia* sp. 1 aff. *E. nordenskioldi* and for the pigmented lineage 2 of this species. Large genomes (2350–3500 Mbp) were found in the rest of the lineages.

Thus, different genetic lineages of the *E. nordenskioldi* have strongly diverged genomes. This could imply that these lineages represent distinct species (Shekhovtsov et al., 2020a, b), or that this is the result of polyploidy in this complex. It is known that *E. nordenskioldi* consists of races with different ploidy: 2*n*, 4*n*, 6*n*, 7*n*, 8*n*, with the chromosome number ranging from 36 to 142–152 (Viktorov, 1997). Diploid chromosome set is believed to be characteristic for the non-pigmented *pallida* form (Viktorov, 1997; Vsevolodova-Perel, Leirikh, 2014). Based on this, it would be reasonable to suggest that the diploid non-pigmented forms are ancestral to this complex. However, transcriptomic data demonstrated (see the Figure) (Shekhovtsov et al., 2020b) that these forms are not at the basis of the tree, and the ancestral forms were pigmented.

Studied specimens

Species/lineage	Location	Genome size, Mbp	SE	*n*
E. nordenskioldi lineage 9	Magadan oblast, Magadan	3284	168	4
Eisenia sp. 1 lineage 1	Novosibirsk oblast, Kiternia	2351	124	4
	Sverdlovsk oblast, Khomutovka	2664	128	4
Eisenia sp. 1 lineage 2	Altai Republic, Biryuzovaya Katun	343	30	4
Eisenia sp. 1 lineage 3	Kemerovo oblast, Kuzedeevo	2746	126	3
	Kemerovo oblast, Zolotoy Kitat	3499	227	4
	Altai krai, Makarievka	2780	9	4
	Novosibirsk oblast, Kiternia	3120	49	3
	Khabarovsk krai, Tigrovoye	3215	43	3
Eisenia sp. 1 *f. pallida* lineage 1	Magadan oblast, Magadan	2494	18	4
Eisenia sp. 1 *f. pallida* lineage 2	Khabarovsk krai, Lesopilnoye	487	3	3
Eisenia sp. 1 *f. pallida* lineage 6	Altai krai, Makarievka	269	28	3

Note. SE – standard error; *n* – number of individuals.



Phylogenetic tree constructed for the *E. nordenskioldi* complex based on transcriptomic data, taken from (Shekhovtsov et al., 2020b).

Grey squares denote the non-pigmented *pallida* form. Numbers near the branches indicate Maximum Likelihood bootstrap support/Bayesian posterior probabilities; asterisks stand for 100/1.0.

Moreover, one of the *pallida* lineages had a large genome while one pigmented lineage had a small one. Therefore, one cannot state that all non-pigmented forms are diploid and pigmented ones are always polyploid. Moreover, the *pallida* form arose independently several times.

The same arguments apply to genome size: it seems more probable that the ancestral genome was large. Moreover, since the majority of *E. nordenskioldi* populations are amphimictic, the ancestor of the complex was amphimictic and diploid. For *Eisenia* sp. 1, the tree topology also implies that large nuclear genome was the ancestral state, and some branches (lineages) subsequently went through genome compaction.

Several populations from diverse geographic locations were sampled for two genetic lineages (lineages 1 and 3 of *Eisenia* sp. 1). Our analysis demonstrated that there is a certain genome size diversity within these lineages, approximately

13 and 27 % for lineages 1 and 3, respectively. It is well known (Viktorov, 1997; Vsevolodova-Perel, Bulatova, 2008) that chromosome number in octaploid *E. nordenskioldi* populations varies widely, and we may suggest a similar mechanism in this case.

Polyploidy results in increased body size in many animals (Otto, 2007). Earthworms, however, may not conform to this pattern: T.V. Malinina and T.S. Perel (1984) found no size differences between *E. nordenskioldi* of different ploidy. Here we could not measure body size, because the studied animals were completely or partially grinded. However, rough estimates suggest that genetic lineages with small genomes were small or average in size (4–7 cm long), while those with large genomes could be either large (to over 10 cm for *Eisenia* sp. 1 lineage 3) or average (5–10 cm for other lineages). Therefore, although we did not observe a clear pattern, we could hypothesize that genome size partially accounts for body size.

Conclusion

In this study we demonstrated that nuclear genome size varies widely among genetic lineages of the *E. nordenskioldi* complex. This corroborates the remarkable differences among them demonstrated by molecular genetic methods. Moreover, there was also some variation between different populations of the same lineage. Both genome expansion and contraction occurred during the evolution of the complex.

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Unusual congenital polydactyly in mini-pigs from the breeding group of the Institute of Cytology and Genetics (Novosibirsk, Russia)

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Abstract. The article describes a new phenomenon in the breeding group of mini-pigs at the Institute of Cytology and Genetics (ICG, Novosibirsk): polydactyly (extra digits), which is unusual because the additional digits are situated at the lateral surface of legs or at the lateral and medial ones. This anomaly was first found here in 2017 in adult animals intended for culling due to incorrect positioning of the legs caused by flexor tendon laxity and resulting in weight-bearing on the palmar surface of the proximal phalanges ("bear's paw"). Therefore, the polydactyly of mini-pigs has a pronounced negative selection effect. A visual survey of the livestock was conducted, and a description of the detected anomaly was compiled. The polydactyly in mini-pigs is a stand-alone trait and is not part of any syndromes. Individuals with polydactyly may have extra digits either on pectoral or on pectoral and pelvic limbs. On thoracic limbs, there may be either one lateral digit or a lateral digit and a medially located rudimentary hooflet. On pelvic limbs, only lateral extra digits can occur. Anatomical and morphological analyses showed that the lateral extra digit is an anatomically complete ("mature") structure, whereas the medial rudimentary digit consists of only a hooflet without other structures characteristic of normal digits. Cytological examination revealed no specific karyotypic features, except for Robertsonian translocation Rb 16;17 previously reported for the mini-pigs of the same livestock. Cytological findings indicated that the polydactyly and Robertsonian translocation are not linked genetically. Genealogical analysis and results of crosses are consistent with a working hypothesis of recessive inheritance of the trait. Overall, the study shows that this type of polydactyly is anatomically and morphologically unique and not typical of *Sus scrofa*. In this species, only polydactyly types with medial accessory toes have been described and are usually inherited as a dominant trait with incomplete penetrance. In our case, the results of test crosses indicate recessive inheritance of the trait with varying expression and incomplete penetrance, because of which poorly expressed phenotypes are not visually detectable.
Key words: polydactyly; multi-fingeredness; lateral and medial position; mini-pigs of ICG SB RAS; recessive inheritance; incomplete penentrance.

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Необычная врожденная полидактилия мини-свиней селекционной группы ИЦиГ СО РАН

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Аннотация. Приведено описание нового для мини-свиней селекционной группы Института цитологии и генетики (ИЦиГ) СО РАН феномена – полидактилии (многопалости). Она отличается от описанных ранее у *Sus scrofa* проявлений этой аномалии тем, что дополнительные пальцы располагаются либо на латеральной стороне конечностей, либо и на латеральной, и на медиальной. Аномалия впервые была обнаружена в 2017 г. у взрослых животных, предназначенных для выбраковки по причине неправильной постановки ног

(«медвежья лапа»). Таким образом, полидактилия мини-свиней ИЦиГ имеет явно выраженный негативный селекционный эффект. Проведено визуальное обследование поголовья и составлено описание этой аномалии. Полидактилия мини-свиней ИЦиГ является изолированной и не входит в состав каких-либо синдромов. Особи с полидактилией могут иметь дополнительные пальцы или на грудных, или на грудных и тазовых конечностях. На грудных конечностях могут присутствовать либо по одному латеральному дополнительному пальцу, либо латеральный палец и медиально расположенное зачаточное копытце. На тазовых конечностях встречаются только латеральные дополнительные пальцы. Анатомо-морфологические исследования показали, что латеральный дополнительный палец – анатомически достаточно полноценная структура, тогда как медиальный зачаток представлен только копытцем без остальных, свойственных нормальным пальцам, структур. Цитологическое исследование не выявило кариотипических особенностей, за исключением ранее описанной для мини-свиней ИЦиГ робертсоновской транслокации Rb 16;17. Впрочем, результаты исследований не указывают на сцепление полидактилии и робертсоновской транслокации. Генеалогический анализ и результаты скрещиваний позволяют принять в качестве рабочей гипотезы предположение о рецессивном наследовании признака. В целом исследование показало, что данная форма полидактилии анатомически и морфологически уникальна и не типична для вида *S. scrofa*, у которого ранее были описаны только формы полидактилии с медиальными дополнительными пальцами, как правило, наследуемые по доминантному типу с неполной пенетрантностью. В нашем случае результаты анализирующих скрещиваний указывают на рецессивное и, возможно, немоногенное наследование признака с варьирующей экспрессией и неполной пенетрантностью, из-за которой слабо выраженные мутантные фенотипы визуально не фиксируются.
Ключевые слова: полидактилия; многопалость; латеральное и медиальное положение; мини-свиньи ИЦиГ СО РАН; рецессивное наследование; неполная пенетрантность.

Introduction

The breeding group of mini-pigs at the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (ICG SB RAS), Novosibirsk, is rather small. Accordingly, there is continuous inbreeding, which results in the appearance of homozygotes for recessive mutations (Nikitin et al., 2014). In late 2017, a new phenomenon was registered in mini-pigs at the ICG SB RAS: polydactyly (extra digits). This anomaly has long attracted the attention of researchers, and in the XVII century its hereditary nature was already identified (Lange, Muller, 2017). Polydactyly can have both atavistic and teratological nature. In the former case, it means complete or partial restoration of a digit(s) lost by the taxon in the course of evolution; in the second, it results from disruptions of normal ontogenesis (Wiesner, Wheeler, 1979). Extra digits may be located on one or more limbs, and their separation from the rest of the digits may be complete or incomplete (Lange, Muller, 2017). It was reported that there is no specific gene that determines the development of a standard or excessive set of digits, but this trait is determined by pleiotropic and polygenic mechanisms as well as various mutations in gene networks that regulate the formation of limbs (Lange, Muller, 2017). Polydactyly may be either a stand-alone abnormal developmental feature (isolated polydactyly) or a sign of a syndrome (syndromic polydactyly) (Gorbach et al., 2010).

The following isolated types of polydactyly are distinguished:

1. Preaxial. The extra digits are located in front of the medial axis of the limb, that is, in front of the first digit (medial position of the extra digit).
2. Postaxial. The other digits are located behind the medial axis, behind the fifth digit (little finger) (lateral position of the extra digit).
3. Central. The rarest, not pre- and not postaxial type.

In *Sus scrofa* pigs, polydactyly was first described more than a hundred years ago (Gorbach et al., 2010). Several types of preaxial polydactyly with incomplete dominance are currently known in this species, including the wild boar (Ptak, 1963; Malynicz, 1982; Gorbach et al., 2010). Preaxial polydactyly with a possible recessive type of inheritance was described relatively recently (Gorbach et al., 2010). It was suggested that this form may be controlled by genes *LMBR*1, *EN*2, *HOXA*10–13, *GLI*3, *WNT*2, *WNT*16, and/or *SHH*, located on porcine chromosome 18 (Gorbach et al., 2010). It is interesting to note that multi-toed feral pigs are common in Cuba and the neighboring islands, where the second digit is divided into two or three (five- or six-toed animals). This anomaly is accompanied by the so-called “bear’s paw” when the animal stands not on two (the norm) but rather on four digits (Ivanchuk, 2011). In addition, polydactyly was also found in Kuban flood-meadow pigs (Kudryavtsev, 1948).

The purpose of this publication is to describe the polydactyly found in 2017 in the breeding group of mini-pigs at the ICG SB RAS.

Materials and methods

The study includes data on 82 individuals from the breeding group of mini-pigs at the ICG SB RAS. Among them:

1) 9 adult animals and 14 newborn piglets, to describe external manifestations of the polydactyly;
2) 2 individuals – a mature sow and a 5-day-old piglet – for anatomical and morphological analyses;
3) 36 adults and 44 newborn piglets from seven litters with the manifestation of polydactyly, to build a genealogical tree.

During visual examination of the piglets with polydactyly, its presence was determined by the number of hooves on the fore and hind limbs of an individual. The anatomical examination was carried out according to generally accepted methods (Glagolev, Ippolitova, 1977; Lebedev, Zelenevsky, 1995). Anatomical examination was carried out according to generally accepted methods. When constructing the genealogical scheme, we assumed a single source of polydactyly: a common ancestor, i. e., for each pair of polydactyly carriers, by tracing the pedigrees in the direction of earlier generations, we found

the most recent common ancestor. Statistical analysis of the results of crosses was carried out by a generally accepted method (Lakin, 1990).

For cytogenetic analysis, four individuals underwent biopsies of the auricle tissue (less than 10 mm in size). From the biopsy material, fibroblast cultures were obtained according to methods of A.S. Graphodatsky et al. (1988) with modifications (Beklemisheva et al., 2016). Suspensions of metaphase cells were prepared from actively dividing cultured fibroblasts by a previously published method (Stanyon, Galleni, 1991). GTG-differential staining was performed according to the standard method (Seabright, 1971).

Results

Visual analysis

Usually, pigs of the *S. scrofa* species have four toes: the 2nd, 3rd, 4th, and 5th and, respectively, four hooves, two of which (the 3rd and 4th) are supporting (Sokolov, 1979). In polydactyly, there should be more than four toes (hooves) on a pig's leg. In mini-pigs at the ICG SB RAS, polydactyly has the following phenotypic characteristics (Fig. 1):

1. No other anomalies accompany it. That is, the polydactyly is isolated.
2. The number of extra hooves on an individual limb is either 1 or 2. Accordingly, the total number of hooves on one limb is either 5 or 6.
3. Extra hooves are present either on the two pectoral limbs or on all four.
4. The extra hooves are symmetrical on a pair of limbs. The number of extra hooves on both thoracic limbs is either 1 and 1 or 2 and 2; on the pelvic limbs, either 1 and 1, or none.
5. With two extra hooves on the forelimbs, a larger claw-like hoof is located laterally, and a much smaller hoof is situated medially.
6. With two extra hooves on both thoracic limbs, there is one lateral extra hoof on the pelvic limbs. With one extra hoof on the thoracic limbs, there are no extra hooves on the pelvic limbs.
7. It is accompanied by a "bear's paw" when an animal with an extra digit stands on all four digits (2nd, 3rd, 4th, and 5th), not on two central digits (3rd and 4th) as is typical of pigs. With extra digits on the pelvic limbs, their incorrect positioning leads to an overgrowth of the hoof horn and lameness.

The polydactyly in the mini-pigs at the ICG SB RAS is characterized by a variation of the size of the lateral extra digits among same-age individuals; visually, the length differs 2–3-fold. In general, the variation of the trait is represented by three distinct phenotypes (see Fig. 1):

1. Lateral extra hooves on the thoracic limbs, whose length in newborns is ~1 mm.
2. Lateral claw-like extra hooves on the thoracic limbs, the length of which in newborns can reach 3 mm.
3. Lateral extra hooves of ≥3 mm size on the thoracic limbs and medial extra hooves in newborns in the form of a horny tubercle ~1 mm high; at the same time, there are lateral extra hooves on the pelvic limbs.

Polydactyly of mini-pigs at the ICG SB RAS is not typical for the species *Sus scrofa*, which has preaxial relics (Malinich, 1982; Gorbach et al., 2010). Animals with the first polydactyly phenotypes have lateral accessory hooves, which can be considered as isolated postaxial polydactyly. For the third phenotype is characterized by the simultaneous presence of lateral and embryonic medial accessory hooves, i.e. polydactyly with two additional toes is both pre- and postaxial.

Anatomical and morphological analysis

When we examined the anatomical material collected from a mature sow with polydactyly of the third phenotypic type, extra digits were visible in the distal part of each of the four limbs. The extra digits were well developed and had a pronounced stratum corneum and an anatomical configuration corresponding to the normally developed digits in pigs but with signs of atrophy. On the 2nd digit of the left hind leg, an overgrown deformed hoof was visible. The rest of the hooves with extra digits had a pathological shape due to an anomaly of the limbs (Fig. 2, *a*).

A radiograph of distal thoracic extremities revealed their tendency to perform the function of additional support (see Fig. 2, *b–d*). Note the relative topographical position of the phalanges of the extra digits on both thoracic limbs. They were found to be located at the distal ends of fifth metacarpal bones, proximal to the first interphalangeal joint of the forelimb's fifth digit. From the lateral surface, the radiograph shows (in an especially clear image of the left forelimb) the pre-

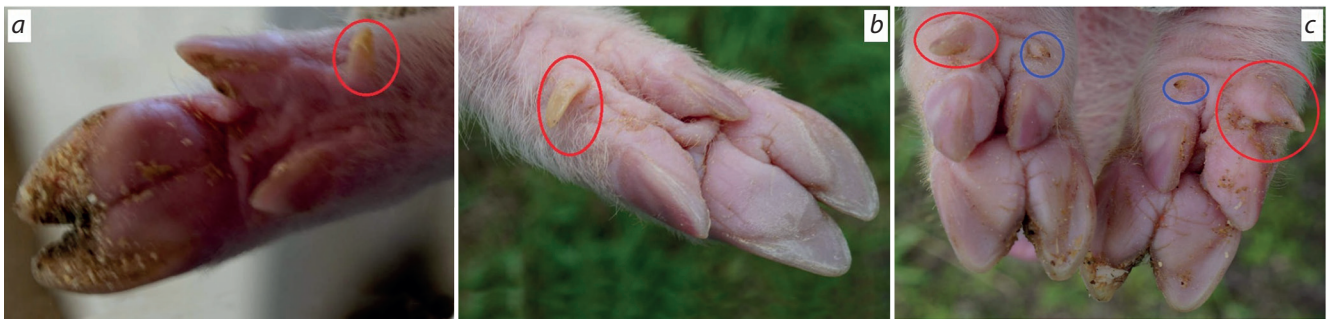

Fig. 1. Illustration of polydactyly phenotypes in piglets among the mini-pigs at the ICG SB RAS.

Lateral extra digits are indicated by red circles, and medial digits are highlighted by blue circles. *a* – the first phenotype; *b* – the second phenotype; *c* – the third phenotype (forelimbs).

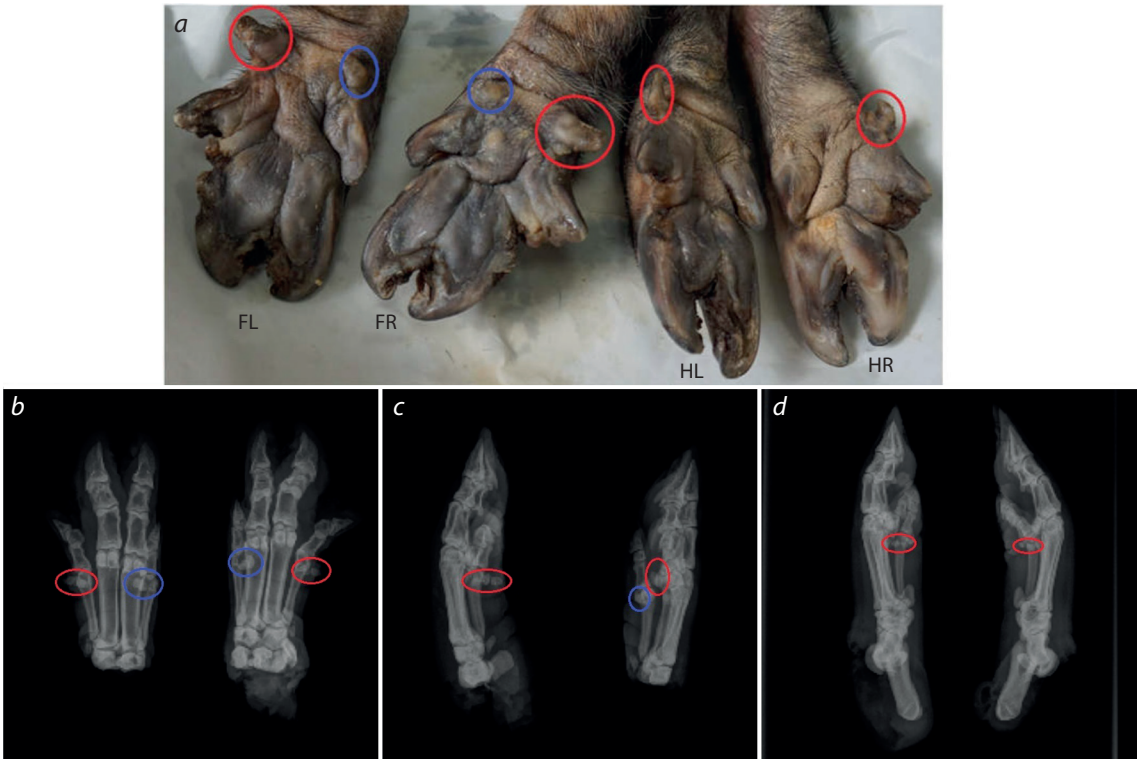

Fig. 2. Limbs of an adult sow of the polydactyly phenotype 3.

Lateral digits are highlighted by red circles; medial digits are highlighted by blue circles (in panels *a*–*c*). Panel *a* shows a photograph of the limbs: FL – front left; FR – front right; HL – rear left; HR – rear right. Panels *b*–*d* present radiographs of the limbs: anterior (left-left and right-right, respectively) in the dorsoventral projection (*b*), anterior in the lateral view (*c*), and posterior at the lateral point (*d*), respectively.

sence of phalanges of the lateral extra digit, with a confirmed anatomical basis for the functions of support, blood supply, innervation, and other trophic functions.

In the examination of a dissected area above the lateral extra digit, a well-formed connective tissue ligament (tendon) was noted on all limbs, which is characteristic of anatomical and topographic structures of porcine distal extremities (Fig. 3). The ligaments of the extra digit consist of smooth fibrous connective tissue with morphological features characteristic of a normal pig limb. Phalanx development with a typically formed metacarpophalangeal joint yielded all the necessary components of a proper joint (synovial fluid, joint ligament, and unity of articular surfaces). The sample demonstrates structural units (common tendon) that determine a possible functional purpose of the extra digit.

External examination of a 5-day-old piglet revealed extra digits in the distal part of thoracic limbs on the lateral surface, which have spiny protrusions with formed horny layers (see Fig. 1, *a* and 4, *a*). Lateral extra digits have a pronounced supporting function, as evidenced by the finding that, five days after birth, there was a noticeable deterioration of the stratum corneum surface. Similar deterioration of the hoof horn on the extra digits also occurred on the limbs of an adult sow (see Fig. 3, *a*). There is a pronounced caudal orientation of the extra digits, opposite to the direction of the four normal ones, which was also found on the pelvic limbs of the adult sow (see Fig. 2, *a*). An extra digit on the lateral side of the limb in both the former and latter cases ends in a claw-like hoof; in the adult sow, it was found to be damaged and blunted at the end (see Fig. 3, *a* and *c*), and in the piglet, the hoof was still sharp (see Fig. 4, *f*). That is, the lateral extra digit serves as an additional support for the "bear's paw".

The preparations of digits from a 5-day-old piglet, including a lateral extra digit, featured a bone base (see Fig. 4, *b*). On the lateral surface separated from the skin, phalanges of the extra digit and a coarse-fibrous-connective-tissue ligament, which is the structural and functional unit of the extra digit, can be observed. On the preparation of the distal limb, the interphalangeal joints of all digits are visible, including the extra digit (see Fig. 4, *c* and *d*). The interphalangeal joint of the extra digit is well-pronounced (see Fig. 4, *e*). The separated dissected extra digit of the thoracic limb from the 5-day-old piglet had all the characteristic morphological features (see Fig. 4, *f*).

Overall, the anatomical analysis of the lateral extra digits from mini-pigs of the ICG SB RAS revealed that in this selection group, a potentially functional structure, i. e., a lateral extra digit with a pronounced bone support base, is present. These data suggest that with a possible load of the limb on it, there is formation of qualitative reference indicators in ontogenesis. On the contrary, the medial extra digit does not have such a supporting bone base and is represented only by the hooflet. Its position in the first digit (thumb) lost in the process of evolution points to incomplete materialization of the second extra digit wherein the extra digit "replaces" the lost thumb.

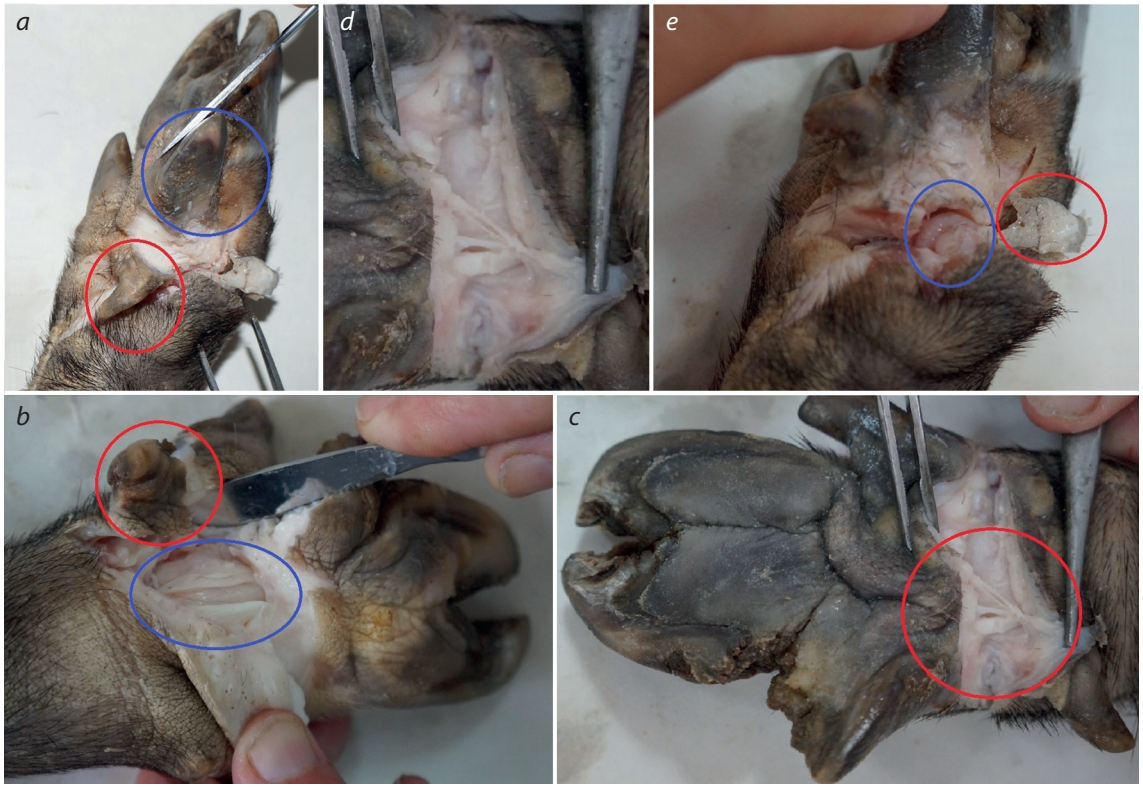

Fig. 3. Analytical preparation of an extra digit from a thoracic limb of an adult female.

a – the first stage of the preparative procedure with the separation of the digit; the wear and tear of the hoof horn is visible on the normal lateral (in a blue circle) and extra (in a red circle) digits; *b* – the second stage of preparation, the histological base (in a blue circle) of the extra digit (encircled in red) is visible; *c* – the third stage of preparation. The tendon (in the red circle) of the ligament of the extra digit is visible; *d* – an enlarged image of the tendon; *e* – dissection of the joint (in the outgrowth indicated by the blue circle) of the extra digit without the horn cover (in the red circle).

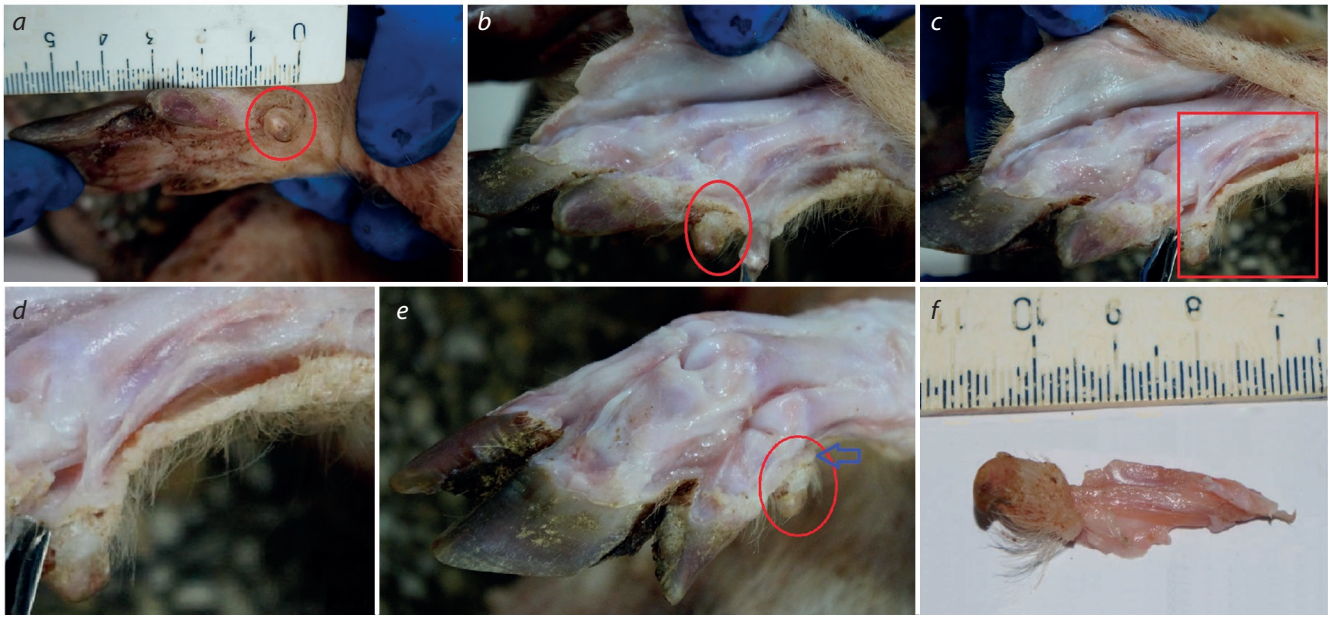

Fig. 4. Anatomical and morphological analyses of the thoracic limbs carrying a lateral extra digit in a 5-day-old piglet from the mini-pigs at the ICG SB RAS.

a – size comparison of standard and extra digits (in a red circle or box); *b* – removal of the skin from the preparation of the limb from a mini-pig with polydactyly (the abnormal lateral digit is in the red circle); *c* – demonstration of the tendons (indicated by the red circle) that attach the extra digit to the bones of the pastern and the phalanx of a normal digit; *d* – an enlarged photo of the same tendons; *e* – demonstration of the bone base of the extra digit on the processed limb of the mini-pig; the blue arrow indicates the joint; *f* – preparation of the extra digit.

Genealogical and genetic analyses

A genealogical scheme based on the principle of the lowest common ancestor of a pair of individuals was constructed to clarify the source of the polydactyly in the breeding group of mini-pigs at the ICG SB RAS. The scheme shows that all probable ascending lines of polydactyly inheritance converge on a common ancestor: boar No. 207 (Fig. 5). Currently available data are insufficient for objective reliable testing of the hypotheses about the inheritance of the trait. The reason is that only a year after the discovery in the breeding stock of individuals with polydactyly, all newborn piglets were examined to register the presence of this anomaly.

From 2018 to 2020, eight litters with polydactyly were obtained: a total of 51 newborns, 14 of them with extra digits. In two litters, where both parents had a normal phenotype (see Fig. 5), six piglets were born, and in each one polydactyly was present. In six other litters, one of the parents had polydactyly, the other one was normal (but had a parent with polydactyly) (see Fig. 5). They gave birth to 39 piglets, of which 12 had extra digits. The genealogical scheme indicates that in all 16 cases of polydactyly, the pedigrees are connected by an ancestor common to the parents of such an individual (see Fig. 5). Based on the pedigrees (Fig. 6), without resorting to statistical analysis, it is already possible to assume recessive inheritance of the polydactyly in the breeding group of mini-pigs.

More information about the genetic nature of this anomaly was given by the outcome of test crosses between i) the pigs that were phenotypically normal but heterozygous for the polydactyly factor(s) and ii) the animals that had extra digits. Statistical analysis of the analytical crossbreeding results (see the Table) revealed that the assumption of monogenic inheritance (in which the offspring would be expected to split according to the phenotype in the ratio of 1:1) is rejected ($\chi^2 = 5.76$, d.f. = 1). Probably, what occurs here is recessive

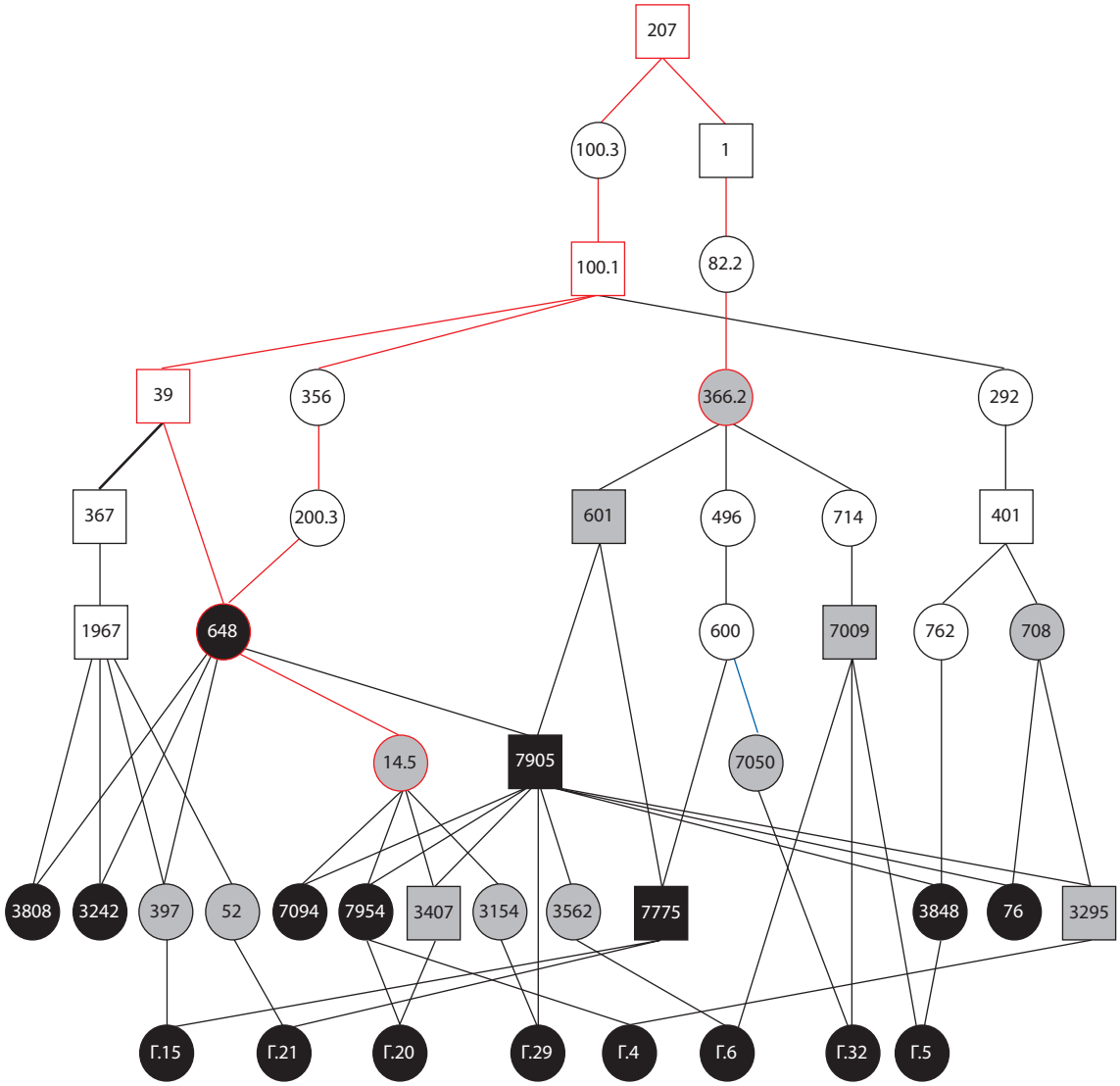

Fig. 5. Scheme of polydactyly transmission pathways in the breeding group of mini-pigs at the ICG SB RAS.

Squares indicate males; circles indicate females; black symbols indicate individuals with polydactyly; grey symbols indicate normal phenotype (the carriage of the trait's genetic factor was confirmed by crosses); white symbols indicate normal phenotype individuals (with unconfirmed carriage of the trait). Red color indicates the ancestors on which the pedigrees of individuals with the manifestation of polydactyly converged and the path between these ancestors.

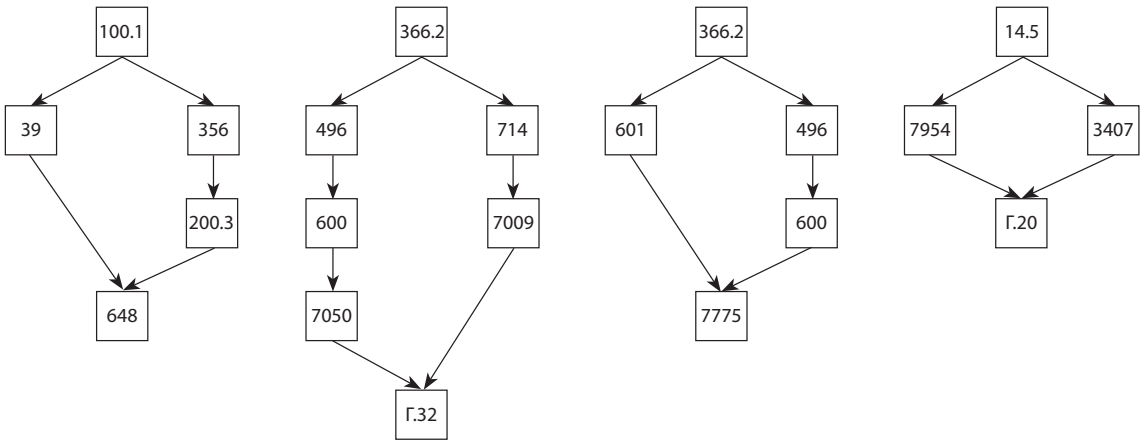

Fig. 6. Fragments of pedigrees of individuals manifesting polydactyly.

IDs of the animals are indicated in squares (gender is not shown). At the top of the pedigrees, the nearest common ancestors are presented, and at the bottom, probands with polydactyly.

The results of backcrosses between pigs with polydactyly and heterozygote for the genetic factor(s) of polydactyly

Number of progenies	Total	Normal	Polydactyly
Real	39.0	27.0	12.0
Expected		19.5	19.5

polygenic (with one or several major genes) inheritance, although it is impossible to rule out a monogenic state with incomplete penetrance, which may cause visually undetectable weak expression of the trait. On the other hand, because the numbers of offspring of the two classes were relatively small, it is necessary to set up other crosses for a more accurate assessment of a larger sample of pigs.

Cytogenetic analysis

To identify specific features of the karyotypes in the breeding group of mini-pigs, this analysis was performed on four individuals. Karyotypes were obtained for the following mini-pigs: two with the normal phenotype (female No. 14.5 and male No. 3407) and two with polydactyly (female No. 3808 and male No. 7905) (Fig. 7).

Based on the GTG-banding data, it was demonstrated that the karyotypes of three studied individuals (IDs 14.5, 3407, and 3808) do not differ from the previously published conventional karyotypes of *Sus scrofa* in the number ($2n = 38$), morphology, and the GTG band pattern of chromosomes (Graphodatsky et al., 2020) (see Fig. 7). In the male with polydactyly (pig No. 7905), a Robertsonian translocation (Rb 16;17, $2n = 37$) was detected, the occurrence of which among the mini-pigs of the ICG SB RAS was reported earlier (Tikhonov et al., 2010), for example, in its male progenitor with polydactyly (pig No. 207).

Discussion

The polydactyly that manifested itself in the breeding group of mini-pigs at the ICG SB RAS is unique for *S. scrofa*. It combines pre- and postaxial types with obvious predominance of the latter. From the point of view of microevolutionary processes, the polydactyly in the mini-pigs at the ICG SB RAS is evidently a new physical feature, namely, the formation of an almost complete lateral extra digit. With maximum expression of this trait, another extra digit, i. e., a rudimentary medial hooflet, is observed at the site of the thumb. In general, *S. scrofa* is characterized by the medial location of the extra digits previously found in some individuals (Malynicz, 1982; Gorbach et al., 2010); the extra digits seem to "replace" the thumb, although anatomically, these "replacements" can differ very significantly from the thumb (Malynicz, 1982; Gorbach et al., 2010). In general, it seems that the very genetic mechanism underlying the formation of the thumb is disrupted in the cases described earlier. Still, information about its location is preserved in the genome.

On the contrary, in the mini-pigs from the ICG SB RAS with five digits, the location of the extra digit with the corresponding fully formed anatomical and morphological structures is genetically determined at the site of the sixth digit (a "second" little finger). When mini-pigs have another type of the anomaly in the form of "six digits" and the extra little toe, there is a rudimentary hoof in place of the first digit (thumb). Still, all the other structures inherent in normal digits are absent here. As a consequence, the rudimentary hooflet of this second extra digit on the six-toed pectoral limb of a mini-pig is located in the first digit (thumb) of the pectoral limb. We believe that this phenomenon requires further anatomical, morphological, and molecular-genetic studies.

Our analysis of karyotypes by standard cytogenetic methods did not reveal any specific features in our mini-pigs with extra digits, except for the Robertsonian translocation Rb 16;17 in one of the four tested animals; this feature was previously identified in this population (Tikhonov et al., 2010).

Candidate genes that may determine polydactyly in pigs are located on chromosome 18 (Gorbach et al., 2010). According to the obtained GTG-banding data, no inter- and intrachromosomal rearrangements involving this chromosome are present in these mini-pigs.

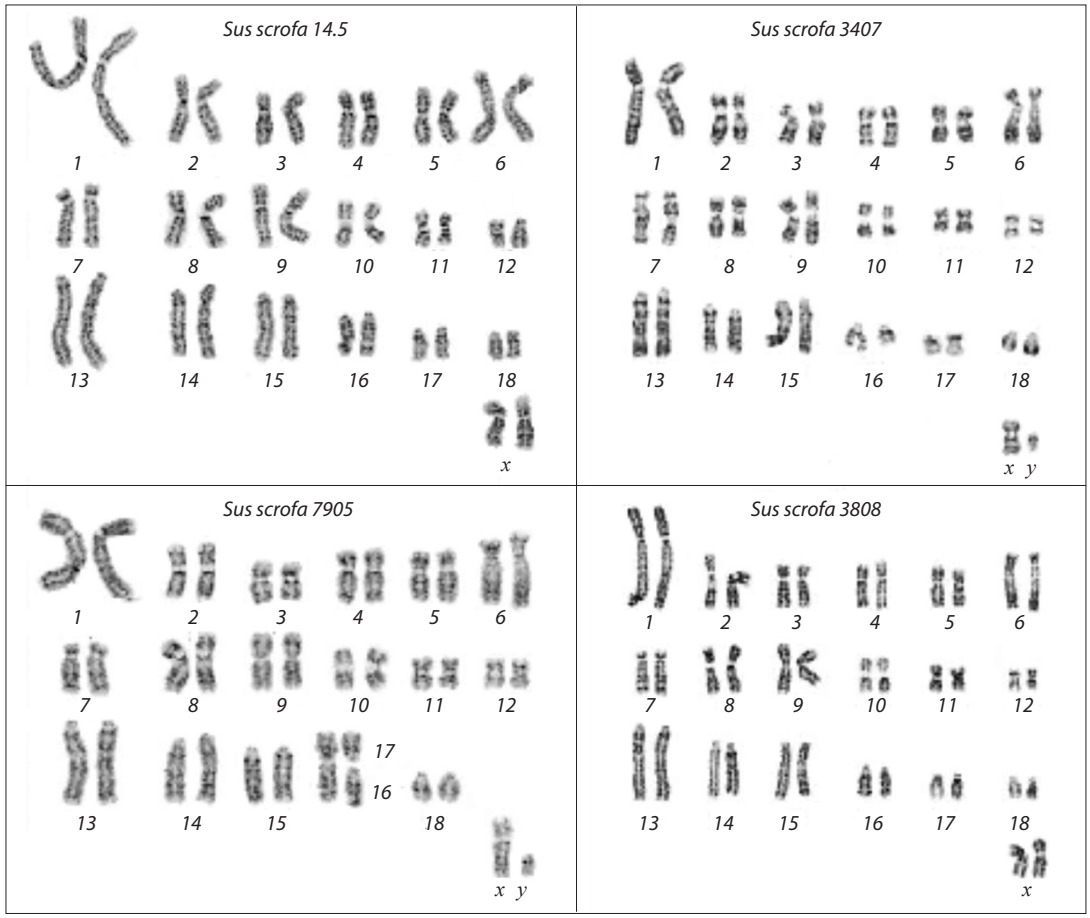

Fig. 7. GTG-stained chromosomes of four individuals in the breeding group of mini-pigs at the ICG SB RAS.

Conclusions

The results of test crosses indicate recessive inheritance of the trait with varying expression and incomplete penetrance, which may also explain why poorly expressed phenotypes are not visually detectable. It is possible that a "bear's paw" without extra digits, which is not noticeable in newborn piglets, may also represent a sort of polydactyly phenotype. In conclusion, it should be noted that the polydactyly in the mini-pigs at the ICG SB RAS has an apparent selection-negative effect. In animals with a "bear's paw", a hoof horn may grow on 3–4 digits, thereby leading to lameness (Fig. 8). Therefore, polydactyly was first detected among the mini-pigs at the ICG SB RAS when we examined animals culled from the breeding stock owing to incorrect leg positioning or lameness.

Fig. 8. An adult mini-pig female with polydactyly and carpal laxity ("bear's paw").

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Negative heterosis for meiotic recombination rate in spermatocytes of the domestic chicken *Gallus gallus*

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Abstract. Benefits and costs of meiotic recombination are a matter of discussion. Because recombination breaks allele combinations already tested by natural selection and generates new ones of unpredictable fitness, a high recombination rate is generally beneficial for the populations living in a fluctuating or a rapidly changing environment and costly in a stable environment. Besides genetic benefits and costs, there are cytological effects of recombination, both positive and negative. Recombination is necessary for chromosome synapsis and segregation. However, it involves a massive generation of double-strand DNA breaks, erroneous repair of which may lead to germ cell death or various mutations and chromosome rearrangements. Thus, the benefits of recombination (generation of new allele combinations) would prevail over its costs (occurrence of deleterious mutations) as long as the population remains sufficiently heterogeneous. Using immunolocalization of MLH1, a mismatch repair protein, at the synaptonemal complexes, we examined the number and distribution of recombination nodules in spermatocytes of two chicken breeds with high (Pervomai) and low (Russian Crested) recombination rates and their F_1 hybrids and backcrosses. We detected negative heterosis for recombination rate in the F_1 hybrids. Backcrosses to the Pervomai breed were rather homogenous and showed an intermediate recombination rate. The differences in overall recombination rate between the breeds, hybrids and backcrosses were mainly determined by the differences in the crossing over number in the seven largest macrochromosomes. The decrease in recombination rate in F_1 is probably determined by difficulties in homology matching between the DNA sequences of genetically divergent breeds. The suppression of recombination in the hybrids may impede gene flow between parapatric populations and therefore accelerate their genetic divergence.
Key words: recombination; heterosis; macrochromosomes; synaptonemal complexes; MLH1.

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Отрицательный гетерозис по частоте мейотической рекомбинации в сперматоцитах домашней курицы *Gallus gallus*

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Аннотация. Преимущества и издержки мейотической рекомбинации являются предметом дискуссий. Поскольку рекомбинация разрушает комбинации аллелей, уже проверенные естественным отбором, и порождает новые с непредсказуемой приспособленностью, высокая частота рекомбинации обычно выгодна для популяций, живущих в быстро меняющейся среде, но не выгодна в стабильной среде. Помимо генетических преимуществ и издержек, существуют цитологические эффекты рекомбинации, как положительные, так и отрицательные. Рекомбинация необходима для синапсиса и сегрегации хромосом. Однако она сопряжена с образованием множества двухцепочечных разрывов ДНК, ошибочная репарация которых может привести к гибели половых клеток или к различным мутациям и перестройкам хромосом. Таким образом, преимуще-

ства рекомбинации (генерация новых комбинаций аллелей) будут преобладать над ее издержками (возникновение вредных мутаций), пока популяция остается достаточно гетерогенной. Используя иммунолокализацию MLH1 белка мисматч-репарации, мы исследовали количество и распределение рекомбинационных узелков в сперматоцитах двух пород кур с высокой (Первомайская) и низкой (Русская хохлатая) частотой рекомбинации и их гибридов F_1 и беккроссов. У гибридов F_1 мы наблюдали отрицательный гетерозис по частоте рекомбинации. Беккроссы на Первомайскую породу были достаточно однородными и имели промежуточную частоту рекомбинации. Различия в общей частоте рекомбинации между породами, гибридами и беккроссами в основном определялись различиями по макрохромосомам. Снижение частоты рекомбинации в F_1, вероятно, обусловлено трудностями в поиске гомологии между последовательностями ДНК генетически дивергентных пород. Подавление рекомбинации у гибридов может препятствовать потоку генов между парапатрическими популяциями и, следовательно, ускорять их генетическую дивергенцию.
Ключевые слова: рекомбинация; гетерозис; макрохромосомы; синаптонемные комплексы; MLH1.

Introduction

Benefits and costs of meiotic recombination are a favorite subject of theoretical discussions and mathematical models (Kondrashov, 1993; Otto, Lenormand, 2002; Hartfield, Keightley, 2012; Rybnikov et al., 2020). They are mostly focused on the population genetic effects of recombination, i. e. its contribution to genetic and phenotypic variability. Crossing over reduces linkage disequilibrium by breaking old allele combinations already tested by natural selection and generating new ones of unpredictable fitness. Therefore, a high recombination rate is generally beneficial for populations living in fluctuating or rapidly changing environments and costly in a stable environment (Otto, Michalakis, 1998; Lenormand, Otto, 2000). Besides genetic benefits and costs, there are cytological effects of recombination, both positive and negative. Recombination is necessary for chromosome synapsis and segregation. However, it involves a massive generation of double-strand DNA breaks. Insufficient or erroneous repair of the breaks leads to the death of the affected germ cells or various mutations and chromosome rearrangements (Zickler, Kleckner, 2015).

Crossing over distribution along the chromosomes is another important variable affecting both genetic and cytological benefits and costs of recombination. Two crossing overs positioned too close to each other do not affect the linkage phase (Gorlov, Gorlova, 2001; Berchowitz, Copenhaver, 2010). Similarly, crossing overs located too close to a centromere of an acrocentric chromosome or to telomere do not produce new allele combinations. In these cases, the cost of recombination is paid, but no benefit is gained. Cytological costs of crossing overs that are too distal or too proximal should also be taken into account. They often lead to incorrect chromosome segregation and generation of chromosomally unbalanced gametes (Koehler et al., 1996; Hassold, Hunt, 2001). Thus, the benefits of recombination (generation of new allele combinations) would prevail over its costs (occurrence of deleterious mutations) as long as the population remains sufficiently heterogeneous.

The heritability of recombination rate was estimated as 0.30 in humans, 0.22 to 0.26 in cattle and 0.15 in sheep (Kong et al., 2004; Sandor et al., 2012; Johnston et al., 2016). Interbreed variation in recombination rate was detected in rams (Davenport et al., 2018) and roosters (Malinovskaya et al., 2019). The most intriguing finding of the latter study was a correspondence between the age of the breed and its recombination rate. Relatively young breeds created by crossing several local breeds showed high recombination rates, while ancient local breeds displayed a low recombination rate. The decrease in recombination rate with breed age might be a correlative response to a decrease in genetic heterogeneity within each breed with time due to inbreeding and artificial selection (Lipinski et al., 2008; Gibbs et al., 2009). Early stages of conscious selection for economic traits were probably accompanied by unconscious selection for a high recombination rate. A reduction of genetic variability, an inevitable result of inbreeding and selection, leads to a decrease in recombination efficiency and therefore reduces selective advantages of high recombination rate.

In this paper, we examine the inheritance of the recombination rate in male F_1 hybrids and backcrosses of the chicken breeds showing the highest (Pervomai) and lowest (Russian Crested) level of recombination among the six breeds examined by L.P. Malinovskaya et al. (2019). The Pervomai breed was produced in 1930–1960 by a complex reproductive crossing of three crossbred breeds: White Wyandotte (derived from crosses between Brahmas and Hamburgs), Rhode Island (derived from crosses between Malays and brown Italian Leghorns) and Yurlov Crower (derived from crosses of Chinese meat chicken, gamecocks and landraces). Russian Crested is an ancient local breed described in the European part of Russia in the early XIX century (Paronyan, Yurchenko, 1989).

We estimated the number and distribution of recombination nodules in spermatocytes using immunolocalization of MLH1, a mismatch repair protein of mature recombination nodules, at the synaptonemal complexes (SCs). This method has proved to produce reliable estimates of the overall recombination frequency and the distribution of recombination events along individual chromosomes (Anderson et al., 1999; Froenicke et al., 2002; Segura et al., 2013; Pigozzi, 2016).

Material and methods

Animals. Thirty-four adult five-month-old roosters were used in this study. Eight of them were Pervomai breed, nine – Russian Crested breed, three – F_1 hybrids between Pervomai dams and Russian Crested sires, fourteen – backcrosses of F_1 sires to Pervomai dams.

The roosters were bred, raised and maintained at the poultry farm of the L.K. Ernst Federal Research Centre for Animal Husbandry under conventional conditions. Maintenance, handling and euthanasia of animals were carried out in accordance with the approved national guidelines for the care and use of laboratory animals. All experiments were approved by the

Ethics Committee on Animal Care and Use at the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (approval No. 35 of October 26, 2016 and 45/2 of January 10, 2019).

Synaptonemal complex spreading and immunostaining. Chromosome spreads were prepared from the right testes by a drying-down method (Peters et al., 1997). Then the slides were subjected to immunostaining according to L.K. Anderson et al. (1999). The slides were incubated overnight in a humid chamber at 37 °C with the following primary antibodies: rabbit polyclonal anti-SYCP3 (1:500; Abcam, Cambridge, UK), mouse monoclonal anti-MLH1 (1:30; Abcam, Cambridge, UK) and human anticentromere (ACA) (1:70; Antibodies Inc., Davis, USA). Secondary antibody incubations were carried out for 1 h at 37 °C. The secondary antibodies used were Cy3-conjugated goat anti-rabbit (1:500; Jackson ImmunoResearch, West Grove, USA), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (1:30; Jackson ImmunoResearch, West Grove, USA) and aminomethylcoumarin (AMCA)-conjugated donkey anti-human (1:40; Jackson ImmunoResearch, West Grove, USA).

Antibodies were diluted in PBT (3 % bovine serum albumin and 0.05 % Tween 20 in PBS). A solution of 10 % PBT was used for blocking non-specific binding of antibodies. Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA, USA) was used to reduce fluorescence fading. The preparations were visualized with an Axioplan 2 microscope (Carl Zeiss, Germany) equipped with a CCD camera (CV M300, JAI Corporation, Yokohama, Japan), CHROMA filter sets and ISIS4 image-processing package (MetaSystems GmbH, Altlußheim, Germany). The location of each imaged immunolabeled SC spread was recorded so that it could be relocated on the slide after FISH.

Fluorescence *in situ* hybridization with BAC probes. After the acquisition of the immunofluorescence signals, the slides were subjected to FISH with universal bird BAC probes CHORY-261 (Damas et al., 2017). Table shows a list of BAC-clones used in this study. BAC DNA was isolated using the Plasmid DNA Isolation Kit (BioSilica, Novosibirsk, Russia) and amplified with GenomePlex Whole Genome Amplification Kit (Sigma-Aldrich Co., St. Louis, MO, USA). BAC DNA was labeled using GenomePlex WGA Reamplification Kit (Sigma-Aldrich Co.) by incorporating biotin-16-dUTP (Roche, Basel, Switzerland).

FISH on SCs was performed following the standard procedure (Liehr et al., 2017). Briefly, 16 µl of hybridization mix contained 0.2 µg of the labeled BAC-probe, 2 µg of Cot-2 DNA of *Gallus gallus* (Trifonov et al., 2009), 50 % formamide in 2xSSC (saline-sodium citrate buffer), 10 % dextran sulfate. Probes were denatured for 5 min at 95 °C and reannealed for 1 h at 42 °C. Synaptonemal complexes spreads were denatured in 70 % formamide in 2xSSC for 3 min at 72 °C. Hybridization was made overnight at 42 °C. Posthybridization washes included 2×SSC, 0.4×SSC, 0.2×SSC (5 min each, 60 °C) followed by 20-min incubation in 4 % dry milk in 4×SSC/0.05 % Triton X-100. All washes were performed at 42 °C in 4×SSC/0.05 % Triton X-100 3 times (5 min each). Hybridization signals were detected with fluorescein avidin DCS and biotinylated anti-avidin D (Vector Laboratories, Inc.).

List of BAC clones used for FISH

BAC clone	*Gallus gallus* chromosome	Arm
CH261-184E5	1	*q*
CH261-44D16	2	*p*
CH261-169K18	3	*q*
CH261-83E1	4	*p*
CH261-2I23	5	*q*
CH261-49F3	6	*q*

Image analysis. We measured the length of each SC and the total SC length in µm, scored the number of MLH1 signals localized on SCs and recorded their positions relative to the centromere using MicroMeasure 3.3 software (Reeves, 2001). For the seven largest macroSCs identified by relative lengths and centromeric indices, we visualized the pattern of MLH1 foci distribution. We divided the average length of SC by intervals and plotted the relative number (the proportion) of MLH1 foci within each interval. To make the intervals on chromosomes of different lengths comparable, we set the number of intervals for each SC proportional to the average SC length, being ~1 µm.

The Statistica 6.0 software package (StatSoft) was used for descriptive statistics. Mann–Whitney U-test was used to estimate the differences between the genotypes in the average number of MLH1 foci per cell and each macrochromosome, $p < 0.01$ was considered to be statistically significant. Values in the text and figures are presented as means ± S.D.

Results

We analyzed the number and distribution of MLH1 foci at 52650 SC in 1350 spermatocytes of 34 roosters. The rooster pachytene karyotype contained 38 autosomal SCs and a ZZ pair. We identified the seven largest macroSCs by their relative lengths and centromeric indices. SC1, SC2 and SCZZ were large metacentrics. They differed from each other in length and centromeric indices ($p < 0.001$). SC3 and SC5 were large and medium-sized acrocentics, while SC4 and SC7 were medium-sized submetacentics, which also differed from each other in their relative lengths and centromeric indices. The macroSCs 6, 8–10 and all microSCs were acrocentric, with gradually decreasing chromosomal sizes (Fig. 1). All chromosomes showed orderly synapsis. No SCs with asynapsis were detected at pachytene spreads of the specimens of the parental breeds and their F_1 hybrids and backcrosses.

In order to test the reliability of the morphological identification of macrochromosomes, we performed FISH with universal BAC probes obtained from the CHORY-261 library, marking chicken macrochromosomes, on SC preparations after immunolocalization of SYCP3 and centromeric proteins (Fig. 2). Comparison of the FISH results with the results of identification by relative sizes and centromeric indices showed good agreement for all chromosomes. We correctly identified the first seven macrochromosomes and chromosome Z. Chromosomes 6 and 7 are of similar SC lengths and are acrocentric and subacrocentric, respectively.

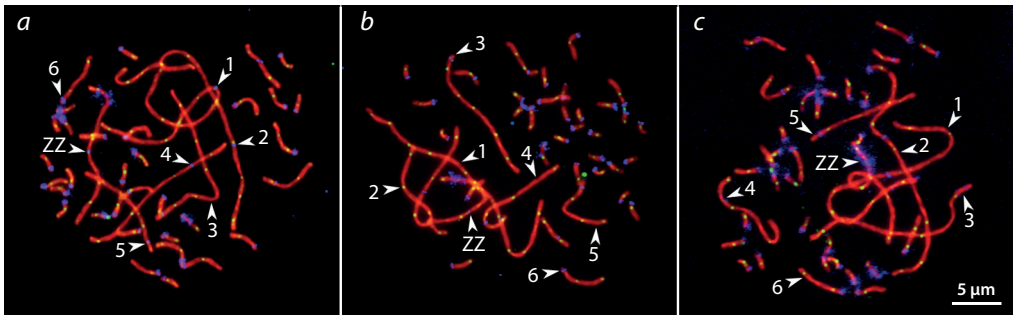

Fig. 1. Pachytene spermatocytes of Pervomai (*a*) and Russian Crested (*b*) and backcross (*c*) roosters after immunolocalization of SYCP3 (red), centromeric proteins (blue) and MLH1 (green).

Arrowheads point to the SCs of the macrochromosomes identified by their lengths and centromeric indices.

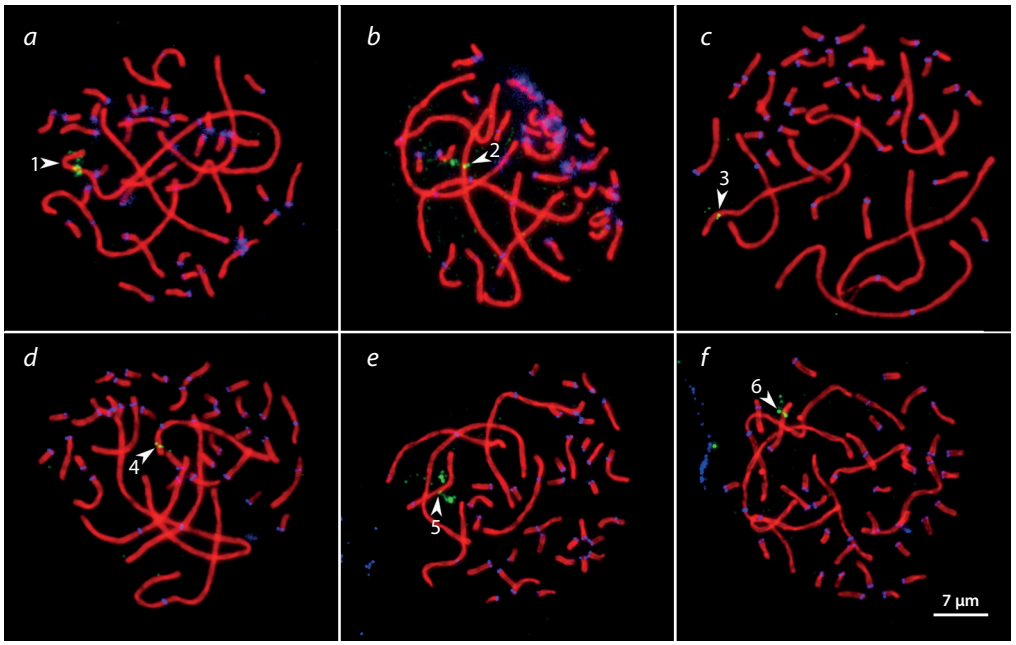

Fig. 2. Pachytene spermatocytes of Pervomai roosters after immunolocalization SYCP3 (red), centromeric proteins (blue) and FISH with universal BAC probes (green) 184E5 (*a*), 44D16 (*b*), CH261-169K18 (*c*), CH261-83E1 (*d*), CH261-2I23 (*e*), CH261-49F3 (*f*).

Arrowheads point to the SCs of the macrochromosomes identified by their sizes and centromeric indices.

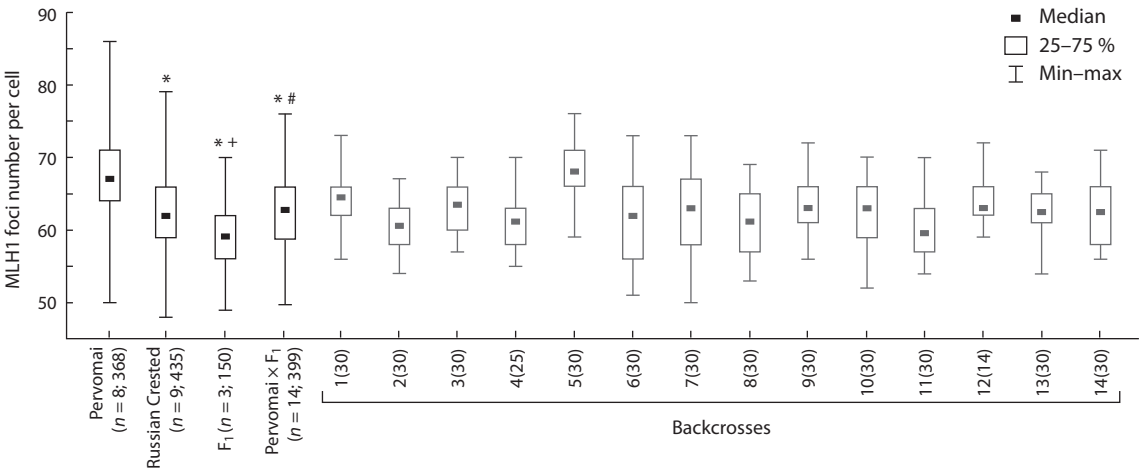

Fig. 3. The number of MLH1 foci per spermatocyte in the roosters of two parental breeds, their F_1 hybrids and backcrosses.

The numbers in parentheses indicate the number of studied individuals and cells. Average values of genotypes are shown in black, individual values of backcrosses are shown in gray. "*" – differences with Pervomai, Mann–Whitney test, $p < 0.01$; "+" – differences with Russian Crested, Mann–Whitney test, $p < 0.01$; "#" – differences with F_1, Mann–Whitney test, $p < 0.01$.

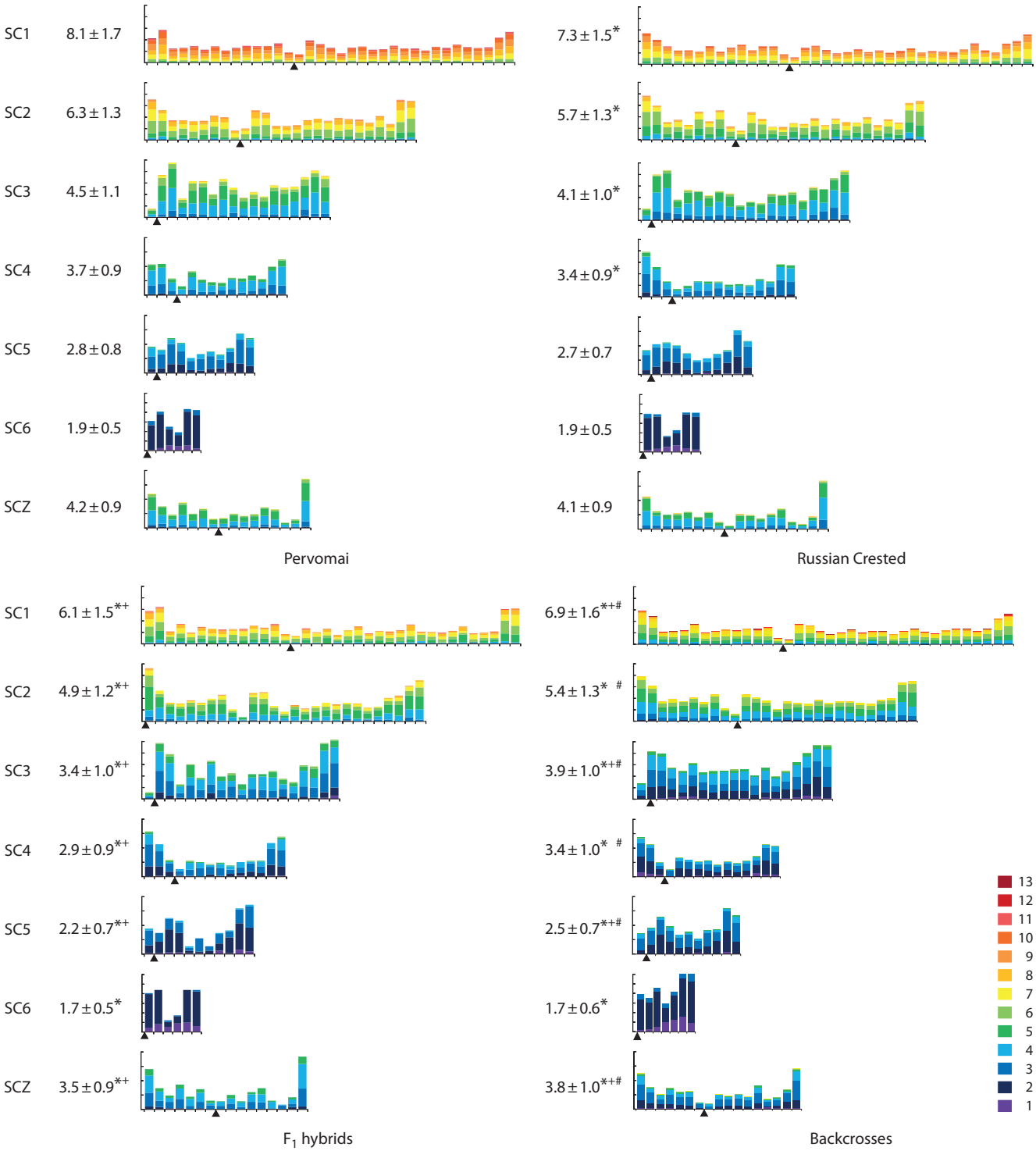

Fig. 4. Average number and distribution of MLH1 foci along the macroSCs of Pervomai, Russian Crested roosters and their F_1 hybrids and backcrosses.

The *X*-axis reflects the position of the foci in the bivalent relative to the centromere (indicated by a triangle). Each interval is equivalent to approximately 1 μm of the SC length. The *Y*-axis reflects the proportion of nodules in each interval. Marks at the *Y*-axis in SC1–SC3 are equal to 0.02, in SC4–SC6, SCZ are equal to 0.05. The colors represent the proportion of bivalents with 1 to 13 MLH1 foci per chromosome. "*" – differences with Pervomai, Mann–Whitney test, $p < 0.01$; "+" – differences with Russian Crested, Mann–Whitney test, $p < 0.01$; "#" – differences with F_1, Mann–Whitney test, $p < 0.01$.

The average number of MLH1 foci per spermatocyte in the first generation hybrids (58.9±0.3) was lower than in both parental breeds: Pervomai (67.3±0.3) and Russian Crested (62.6±0.3). The differences between hybrids and both parental breeds are significant (Mann–Whitney U-test is 11.4 and 14.2, respectively; $p < 10^{-6}$). The backcrosses were homogeneous for the number of MLH1 foci (Fig. 3). They demonstrated a low average MLH1 foci number (62.6±0.5), typical for the Russian Crested ($p = 0.80$), although they exceeded F_1 hybrids in this trait ($p < 10^{-6}$). These results indicate negative

heterosis of the recombination rate measured as MLH1 foci number per pachytene cell.

These differences between parental breeds were mainly determined by the four largest macrochromosomes (Mann–Whitney test, $p < 0.01$) (Fig. 4). SCZZ, SC5 and SC6 of the F_1 hybrids contained fewer MLH1 foci than the corresponding macroSCs of the parental breeds, Pervomai and Russian Crested (Mann–Whitney test, $p < 0.01$). In the backcrosses to Pervomai, the number of MLH1 foci on the SC of ZZ and the six largest autosomes remained significantly smaller than on the corresponding SCs of Pervomai (Mann–Whitney test, $p < 0.01$). However, it was significantly higher on all SCs but SC6 than in the F_1 hybrids (Mann–Whitney test, $p < 0.01$) (see Fig. 4).

Despite these differences in the number of MLH1 foci per particular macrochromosome between the parental breeds, F_1 and backcrosses, each of them showed almost the same chromosome-specific pattern of MLH1 foci distribution along the SC (see Fig. 4). On most chromosomes, an increase in the frequency of recombination was observed in the distal regions.

Discussion

The most important and surprising result of our study is a discovery of overdominance of low recombination rate in F_1 hybrids, measured as the number of MLH1 foci per pachytene cell. Backcrosses of the F_1 hybrids to the parental breed with high recombination rate were rather homogenous and showed an intermediate recombination rate. Thus, the model of inheritance of recombination rate in roosters can be formally described as negative heterosis in F_1 and additive inheritance in backcrosses.

The differences in overall recombination rate between the breeds, hybrids and backcrosses were mainly determined by the differences in the crossing over number in the large macrochromosomes. They are characterized by a high (up to 13!) and variable number of crossing overs, while small macrochromosomes have one or two chiasmata and each microchromosome contains only a single obligate chiasma necessary for orderly chromosome segregation.

Generally, crossbreeds are expected to show positive heterosis for productivity traits (hybrid vigor) (Chen, 2013). This expectation contradicts the negative heterosis for the recombination rate observed in this study. Interestingly, the rate of dilution of heterosis for recombination rate in backcrosses is higher than the rate of dilution of positive heterosis for economic traits, at least in plants (Fridman, 2015). The decrease in recombination rate in F_1 is probably determined by difficulties in homology matching between the DNA sequences of genetically divergent breeds (which we shall discuss below), rather than by dominant/overdominant genetic effects. With further level of backcrossing, the recombination rate acts like a regular complex trait with additive heritable component and environmental influence.

Our finding poses at least three interesting questions. How common is the negative heterosis for the recombination rate? What might be its molecular mechanism? What are its population genetic implications?

The first question is difficult to answer because we are aware of only a few prior studies in which recombination rates have been compared between parental breeds or species and their hybrids. There were no significant differences in autosomal recombination rate between two species of dwarf hamsters diverged about 1 MYA and their F_1 female and male hybrids (Bikchurina et al., 2018). On the other hand, recombination in female hybrids between *Microtus arvalis* and *M. levis* diverged from 0.2 to 0.4 MYA and differing by a series of chromosomal rearrangements was significantly reduced compared to the parental species (Torgasheva, Borodin, 2016). Interspecific hybrids between *Saccharomyces cerevisiae* and *S. paradoxus* demonstrated low frequencies of genetic recombination (Hunter et al., 1996). Genome-wide introgression between two closely related nematode species *Caenorhabditis briggsae* and *C. nigoni* also revealed substantial suppression of recombination in the hybrids (Bi et al., 2015).

The molecular mechanism of negative heterosis for recombination rate is probably linked with the initial stages of chromosome synapsis and recombination, which includes scheduled generation of multiple double-strand DNA breaks (DSB), RAD51-mediated strand invasion and sequence homology matching (Zickler, Kleckner, 2015). Reduced recombination in interspecies hybrids may occur due to a significant decrease in homology between parent species accompanied by serious impairments of the chromosome synapsis in meiosis. However, even a minor decrease in homology at the early stages of divergence can apparently affect recombination due to decreased sequence identity. Comparison of recombination boundary sequences suggests that recombination in hybrids may require a region of high sequence identity of several kilobases in length (Ren et al., 2018).

Similarly, the study of recombination rate in hybrids between *S. cerevisiae* strains using high-throughput method showed a positive correlation of its level with sequence similarity between homologs at different scales (Raffoux et al., 2018). This is consistent with the finding that sequence divergence greater than about 1 % leads to the suppression of recombination due to heteroduplex rejection by the mismatch repair machinery (Chen, Jinks-Robertson, 1999). An anti-recombination activity of the mismatch repair system during meiosis might contribute towards a decrease in recombination rate in hybrids between diverging breeds, populations and species (Radman, Wagner, 1993). At relatively low genetic distances it decreases the recombination rate in the hybrids, at greater genetic distances it impairs chromosome synapsis and might lead to hybrid sterility due to meiotic silencing of unpaired chromatin (Turner, 2015).

Conclusion

There might be interesting evolutionary and population genetic implications of our findings. The negative heterosis for recombination in the hybrids may play an important role in speciation. Suppression of recombination impedes gene flow between parapatric populations and therefore accelerates their genetic divergence (Rieseberg et al., 1999; Baack, Rieseberg, 2007). A possibility of negative heterosis for recombination may also be taken into account in the calculations of the introgression time based on the size of linkage disequilibrium blocks (Payseur, 2010). They are based on the assumption that global and local recombination rates are constant over the

generations. Our data indicate that it might not be the case. We detected a decrease in recombination in the macrochromosomes of the hybrids, while the microchromosomes retained the same recombination rate because it had already been the minimal required for orderly segregation.

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Influence of leptin administration to pregnant female mice on obesity development, taste preferences, and gene expression in the liver and muscles of their male and female offspring

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Abstract. The consumption of food rich in sugar and fat provokes obesity. Prenatal conditions have an impact on taste preferences and metabolism in the adult offspring, and this impact may manifest differently in different sexes. An increase in blood leptin level in pregnant females reduces the risk of obesity and insulin resistance in the offspring, although the mechanisms mediating this effect are unknown. Neither is it known whether maternal leptin affects taste preferences. In this study, we investigated the effect of leptin administration to pregnant mice on the development of diet-induced obesity, food choice, and gene expression in the liver and muscles of the offspring with regard to sex. Leptin was administered to female mice on days 11, 12, and 13 of pregnancy. In male and female offspring, growth rate and intake of standard chow after weaning, obesity development, gene expression in the liver and muscles, and food choice when kept on a high-calorie diet (standard chow, lard, sweet cookies) were recorded. Leptin administration to pregnant females reduced body weight in the female offspring fed on the standard diet. When the offspring were given a high-calorie diet, leptin administration inhibited obesity development and reduced the consumption of cookies only in males. It also increased the consumption of standard chow and the mRNA levels of genes for the insulin receptor and glucose transporter type 4 in the muscles of both male and female offspring. The results demonstrate that an increase in blood leptin levels in pregnant females has a sex-specific effect on the metabolism of the offspring increasing resistance to obesity only in male offspring. The mechanism underlying this effect includes a shift in food preference in favor of a balanced diet and maintenance of insulin sensitivity in muscle tissues.
Key words: leptin; taste preferences; obesity; developmental programming.

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Влияние введения лептина беременным самкам мышей на развитие ожирения, вкусовые предпочтения и экспрессию генов в печени и мышцах у потомства разного пола

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Аннотация. Потребление сладкой и жирной пищи способствует развитию ожирения. Условия пренатального развития влияют на вкусовые предпочтения и метаболизм в зрелости, и это может по-разному проявляться в зависимости от пола. Показано, что повышение уровня лептина в крови беременных самок снижает риск развития ожирения и инсулинорезистентности у потомства, однако механизмы его действия на чувствительность к инсулину у потомства не установлены. Неизвестно также, влияет ли материнский лептин на вкусовые предпочтения. Задачей настоящего исследования было изучение влияния введения лептина беременным самкам мышей на развитие ожирения, индуцированного диетой, вкусовые предпочтения и экспрессию генов в печени и мышцах у потомства в зависимости от пола. Оценивали влияние введения лептина самкам мышей на 11, 12 и 13-й день беременности на рост и потребление пищи в стандартных условиях, развитие ожирения, выбор компонентов пищи и экспрессию генов в печени и мышцах при содержании на высококалорийной диете (стандартный корм, свиное сало, сладкое печенье) у потомства разного пола.

Введение лептина беременным самкам снижало вес тела у женского потомства на стандартной диете. При содержании потомства на высококалорийной диете введение лептина тормозило развитие ожирения и снижало потребление печенья только у самцов, а также повышало потребление стандартного корма и уровень мРНК генов инсулинового рецептора и переносчика глюкозы четвертого типа в мышцах у потомства обоего пола. Результаты демонстрируют, что повышение лептина в крови беременных самок оказывает зависящее от пола влияние на метаболизм потомства, увеличивает устойчивость к развитию ожирения только у потомства мужского пола и механизм этого влияния включает в себя смещение вкусовых предпочтений в пользу сбалансированного корма и поддержание чувствительности мышц к инсулину.
Ключевые слова: лептин; вкусовые предпочтения; ожирение; программирование развития.

Introduction

Nowadays, one of the main causes of the widespread occurrence of obesity and conditions associated therewith is the consumption of high-calorie food (Astrup et al., 2008). The choice of food considerably depends on taste preferences (Duffy et al., 2009), and the preference to palatable fatty and sweet (obesogenic) food contributes to the epidemic prevalence of obesity (May, Dus, 2021; Spinelli et al., 2021).

Taste preferences and tendency to metabolic impairments in an individual are determined by the genotype (Chmurzynska, Mlodzik, 2017; Diószegi et al., 2019) and early development conditions (Mezei et al., 2020). The undernourishment, overnutrition, obesity, and diet of pregnant and nursing dams may exert deferred effects on the taste preferences and metabolic phenotype of the offspring in adulthood and thereby increase or decrease the risk of obesity (Barker, Osmond, 1986; Ong et al., 2012; Gabory et al., 2013; Bale, 2015). It is important to investigate the maternal environment factors that modulate offspring development and the molecular and physiological mechanisms targeted by these factors. Understanding them may add to the elaboration of methods that would correct development in order to reduce the risk of metabolic impairments.

Leptin, a hormone produced in the adipose tissue, is considered to be a programming factor of the maternal environment. Model experiments indicate that high maternal leptin levels in the pregnancy reduce body weight, increase insulin sensitivity, and improve metabolic characteristics in the adult offspring fed on balanced or high-calorie diets, and this effect may manifest differently in different sexes (Pennington et al., 2012; Makarova et al., 2013; Pollok et al., 2015; Talton et al., 2016; Denisova et al., 2020). Nevertheless, molecular mechanisms mediating the programming action of maternal leptin remain obscure.

Maternal leptin may exert a deferred effect on metabolic processes in the liver and muscles of the offspring. Developmental programming is thought to be associated with changes in gene expression in the offspring via epigenetic modifications induced by maternal environmental factors (Laker et al., 2014). It is unknown whether leptin affects the expression of genes encoding regulatory factors and enzymes responsible for carbohydrate and lipid metabolism in the liver and muscles of the offspring. Maternal leptin may also contribute to a lower predisposition to diet-induced obesity in the offspring via food choice (Pollock et al., 2015), but this issue has been poorly studied. The diet components (fats, proteins, or sugars) the consumption of which may be affected by maternal leptin are unknown. Neither is it known whether the programming action of maternal leptin on the offspring depends on sex or what mechanisms mediate the programming effect of maternal leptin on food choice in the offspring.

The goal of this study was to investigate the effect of leptin administration to pregnant female mice on metabolic indices, taste preferences, and the expression of genes in the liver and muscles of the offspring with regard to sex.

Materials and methods

Experimental animals. The European Parliament Directive 2004/10/EC on the principles of good laboratory practice and the Russian regulations for accommodation and care of animals, GOST 33215-2014, were followed.

Experiments were conducted with C57BL/6J mice housed at the vivarium of the Institute of Cytology and Genetics, Novosibirsk, Russia. The animals were kept at 12-h daylight with free access to water and standard chow for the conventional maintenance and breeding of rodents (BioPro Company, Novosibirsk, Russia). Mature females were mated to males of the same strain. The mating was judged from the presence of a copulation plug. The appearance of the plug signified day 0 of pregnancy. The females were administered 0.2 mg/kg of recombinant murine leptin (Peprotech, United Kingdom) or the same volume of normal saline on days 11, 12, and 13 of pregnancy. The injections were done subcutaneously in the shoulder area. The female weight and amount of consumed food were recorded daily. The delivery dates and litter sizes were recorded. Large litters (> 7) were reduced to 7 on day 1 after delivery by discarding pups of the lowest weights. The dam and pups were weighted on days 0, 7, 14, 21, and 28 after delivery. The offspring were separated from their mothers on day 28 after birth.

To assess the effect of maternal leptin on the metabolic indices of the offspring in the postnatal life, one male and one female from each litter were kept individually. The female offspring of dams having received normal saline included 6 animals, and the male, 5. The female offspring of dams having received leptin was 8 animals, and so was the male offspring. The animals were weighted on a weekly basis, and the amount of food eaten weekly was assessed. At the age of 10 weeks, the mouse diet was supplemented with obesogenic food: sweet cookies and pork lard. Mice were being fed on this diet for 10 weeks and were weighted weekly. The standard chow was replaced once a week, and cookies and lard were replaced three times a week. The following parameters were recorded: the amounts of daily eaten standard chow, cookies, and lard; the amounts of energy consumed with these kinds of food (lard, 8 kcal/g; standard chow, 2.5 kcal/g;

cookies, 4.58 kcal/g); the overall amount of consumed energy normalized to body weight; and the energy consumed with each kind of food as a percentage of the entire energy consumed.

At the end of the experiment, mice were euthanized by decapitation. Muscle and liver tissue samples were frozen and stored in liquid nitrogen to measure the rate of gene expression.

Diet. Standard chow was purchased from BioPro, Novosibirsk, Russia. Composition: two-component grain mixture, milk components, high-protein components (vegetable and animal proteins), vegetable oil, amino acids, organic acids, vitamin-mineral premix, and fiber. Crude protein: 22 %. Energy value 2500 kcal.

Pork lard and cookies were bought in a food store. Cookie composition (g/100 g): proteins – 6.9, fats – 18.4, carbohydrates – 71.8. Energy value 458 kcal/100 g. Lard (subcutaneous fat): proteins – 1.8, fats – 94.2, carbohydrates – 0. Energy value 800 kcal/100 g.

Assay of mRNAs. Levels of mRNAs were assessed by reverse transcription followed by relative quantitation real-time PCR. Total RNA was isolated from tissues with an ExtractRNA kit (Evrogen, Moscow, Russia) according to manufacturer's recommendations. Reverse transcription was conducted with MMLV reverse transcriptase (Evrogen) and oligo-dT primer according to manufacturer's recommendations.

PCR was conducted with the qPCRmix-HS LowROX reaction premix (Evrogen) and the TaqMan Gene Expression Assay system for mouse genes (Applied Biosystems): *Insr*, Mm01211875_m1; *Fgf21*, Mm00840165_g1; *G6pc*, Mm00839363_m1; *GCk*, Mm00439129_m1; *Ppargc1a*, Mm01208835_m1; *Pklr*, Mm00443090_m1; *Acaca*, Mm01304257_m1; *Pnpla2*, Mm00503040_m1; *Igf*, Mm00439560_m1; *Slc2a4*, Mm00436615_m1; and *Actb*, Mm00607939_s1.

The expression rates of the following genes were assayed in the liver: *InsR* for insulin receptor, *Gck* for glucokinase, *Pklr* for pyruvate kinase, *G6pc* for glucose-6-phosphatase, *Pnpla2* for triacylglyceride lipase ATGL, *Acaca* for acetyl Co-A carboxylase, *Pgc1* for peroxisome proliferator-activated receptor γ coactivator PPARGC1A, *Fgf21* for fibroblast growth factor 21, and *Igf1* for insulin-like growth factor 1. In muscles: *Slc2a4* for glucose transporter GLUT4 and *InsR*. The *Actb* gene for β-actin was used for reference.

The results were statistically evaluated with Statistica 10.0. Descriptive statistics was used in the calculation of group means and standard errors of the mean. Body weight and food consumption in pregnant females and their offspring were analyzed by repeated measures ANOVA with the assessment of the factors "experimental treatment" (administration of leptin or normal saline) and "day of pregnancy" for pregnant females; factors "sex" (males or females), "experiment" (administration of leptin of normal saline to mothers), and "age" (4 to 10 weeks) for the offspring fed on standard diet; factors "sex", "experiment", and "age" (10 to 20 weeks) for the offspring fed on the obesogenic diet. Intergroup differences were assessed by the Newman–Keuls post-hoc test. The effects of sex and the prenatal factor on gene expression in the liver and muscles were assessed by two-way ANOVA with the "sex" and "experiment" factors. Intergroup differences were assessed with Student's *t* test. The results are shown in plots as mean ± SEM.

Results

Influence of leptin administration on food consumption and body weight in pregnant females

Leptin administration to female mice on pregnancy days 11, 12, and 13 had no significant impact on body weight (Fig. 1, *a*), although it reduced food intake by 20 % ($p < 0.001$, repeated measures ANOVA, factors "experiment" × "day of pregnancy" (see Fig. 1, *b*). The anorectic action of leptin lasted for no more than 24 h, as the amounts of food consumption by females having received leptin and normal saline aligned on day 2 after the last injection.

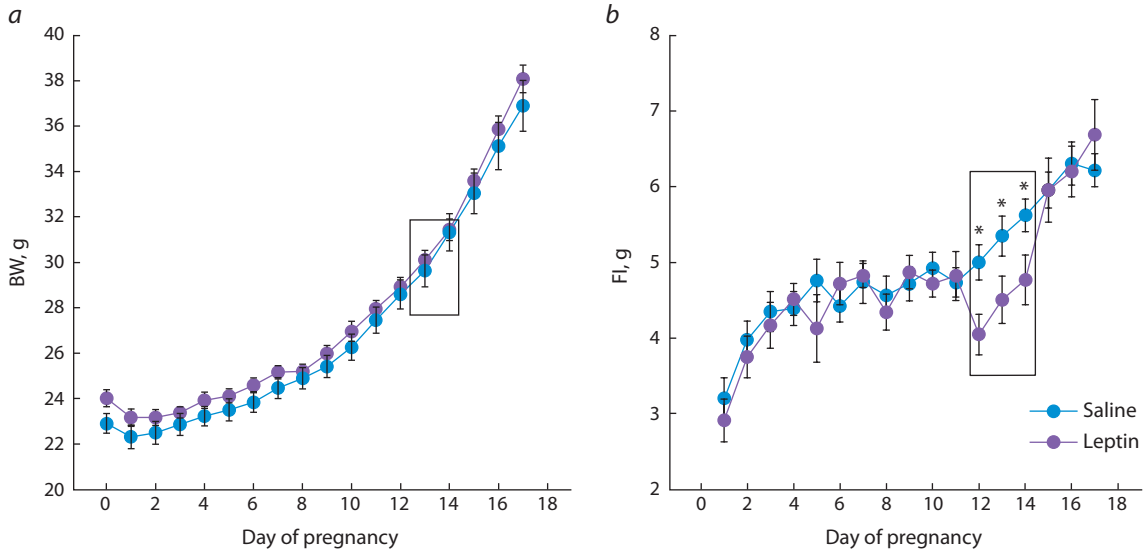

Fig. 1. Influence of leptin administered to female mice on pregnancy days 11, 12, and 13 on body weight (*a*) and food intake (*b*). Data are presented as mean ± SEM. $^{*}p < 0.05$, Student's t test.

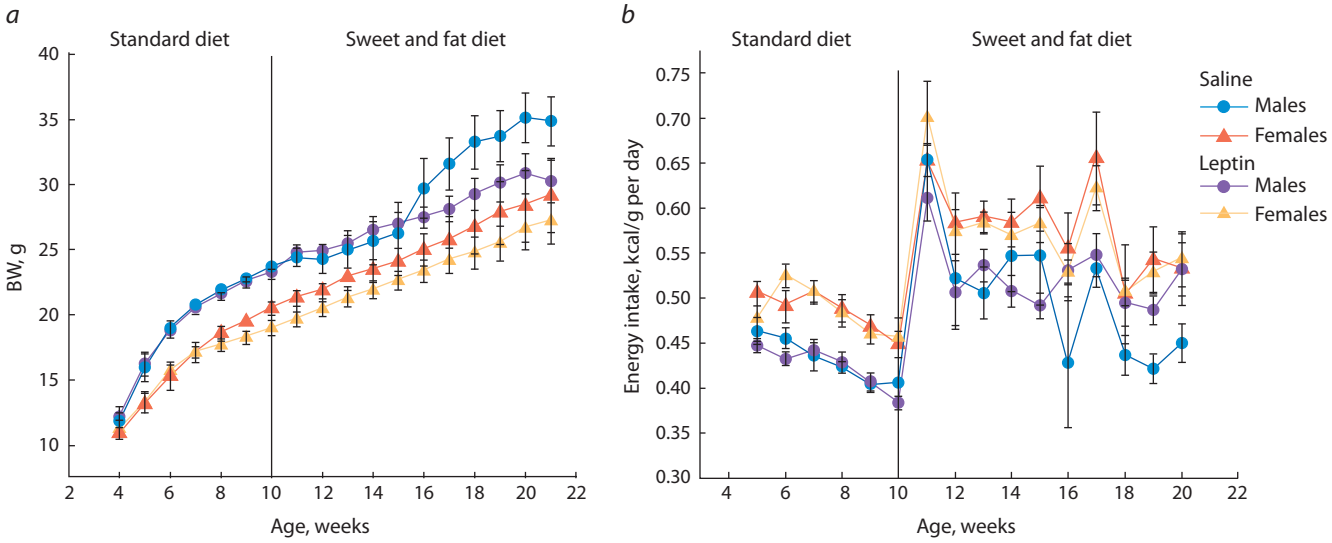

Fig. 2. Influence of leptin administration to pregnant female mice on body weight (*a*) and the ratio of consumed energy and body weight (*b*) in the male and female offspring receiving the standard and obesogenic diet.

Influence of leptin administered to female mice on body weight and the consumption of standard chow in the male and female offspring

When nursed, the male and female offspring did not differ in weight. Leptin administration to pregnant dams had no significant effect on offspring weight (data not shown).

The dynamic patterns of weight after weaning onto standard chow were different in males and females. Within the first week of feeding on standard chow, males overweighed females and weighed more throughout the experiment ($p < 0.0001$, $F_{1.24} = 30.32$, "sex", "sex" × "age" $p < 0.00001$, $F_{6.144} = 8.23$, repeated measures ANOVA). We performed repeated measures ANOVA with the "experiment" and "age" (weeks 4–10) factors in males and females separately.

The male offspring whose mothers had received leptin did not differ in weight from the male offspring of control females when kept singly and fed on standard chow in weeks 4–10. The female offspring of leptin-receiving females did not differ from the female offspring of control females till sexual maturity (8 weeks), but then they lagged behind females born in the control group, and this trend remained throughout the experiment (Fig. 2, *a*). Females consumed more energy per body weight unit than males ($p < 0.0001$, $F_{1.24} = 34.1$, factor "sex", repeated measures ANOVA). Leptin administration to mothers during pregnancy did not influence this index (see Fig. 2, *b*).

Body weight and food consumption in the male and female offspring fed on obesogenic diet

Repeated measures ANOVA revealed no effect of the "experiment" factor on the female offspring in the analysis of body weights of males and females fed on obesogenic diet within 10 weeks. However, it showed a significant interaction between the "age" and "experiment" factors ($p < 0.01$, $F_{9.90} = 2.82$) in the male offspring. The male offspring of dams having received normal saline or leptin did not differ in body weight, but then the males born in the control group began to gain weight sharply and outperformed the males born to leptin-receiving dams (see Fig. 2, *a*). In the females born to control and leptin-receiving dams, the difference arising in keeping on standard chow remained when they received the obesogenic diet (see Fig. 2, *a*).

Energy consumption normalized to body weight increased dramatically when lard and cookies were added to the diet (see Fig. 2, *b*). It remained higher in females than in males ($p < 0.01$, $F_{1.22} = 9.06$, factor "sex", repeated measures ANOVA).

To assess the influence of maternal leptin on taste preferences in the offspring, the contributions of each diet component (standard chow, lard, and cookies) to the overall energy consumption were analyzed. Pronounced sex differences in the consumption of standard chow (males ate more than females, $p < 0.001$, $F_{1.22} = 34$, factor "sex") and cookies (males ate less than females, $p < 0.01$, $F_{1.22} = 12.2$, factor "sex") were recorded. No significant difference between males and females in lard consumption was noted (Fig. 3).

Leptin administration to pregnant mice affected taste preferences in the offspring, and this effect was more pronounced in males. Maternal leptin increased the share of standard chow in the overall energy consumption in the offspring of both sexes within the first five weeks of receiving the obesogenic diet ($p < 0.05$, $F_{1.22} = 4.03$, factor "experiment", repeated measures ANOVA), but in males the effect was greater than in females (see Fig. 3, *a*). Maternal leptin lowered the share of cookies only in males throughout the time of being fed on obesogenic diet ($p < 0.05$, $F_{1.22} = 5.3$, factor "experiment", repeated measures ANOVA, Fig. 3, *b*) and had no impact on lard consumption regardless of sex (see Fig. 3, *c*).

Expression of genes involved in carbohydrate and lipid metabolism in the liver and muscles

The expression rates of the following genes were assayed in liver tissue: *InsR* for insulin sensitivity, *Gck* and *Pklr* for glycolysis enzymes, *G6pc* for gluconeogenesis, *Pnpla2* for lipolysis (adipose triglyceride lipase), *Acaca* for lipogenesis, and genes encoding regulatory factors that affect metabolism

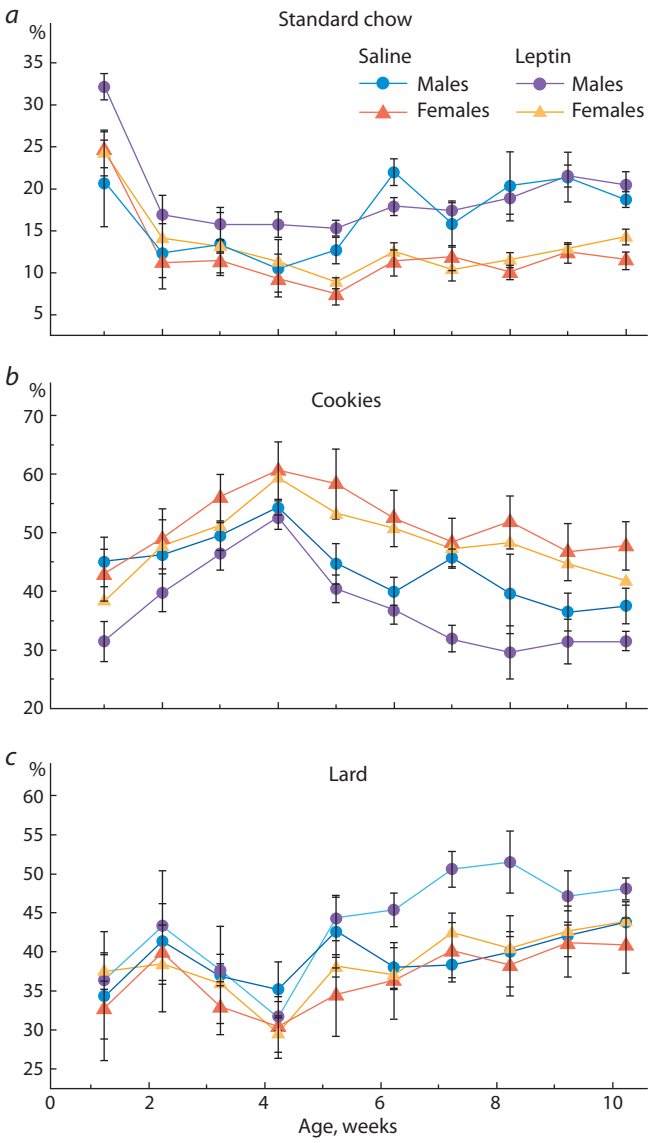

Fig. 3. Influence of leptin administered to pregnant mice on the percentages of energy consumed with standard chow, cookies, and lard in the male and female offspring fed on the obesogenic diet.

in the liver (*Pgc1*) or are involved in the regulation of carbohydrate and lipid metabolism in the entire body (*Fgf21* and *Igf1*). Leptin administration to pregnant mice did not affect the expression of these genes in the liver. A significant influence of sex on the expression of the *Fgf21* gene for fibroblast growth factor 21 was noted. Its mRNA level was lower in females than in males ($p < 0.05$, $F_{1.18} = 5.4$, 2-way ANOVA, Fig. 4, *a*).

In muscles of both sexes, leptin administration to mothers caused higher expression rates of the genes *InsR* for insulin receptor and *Slc2a4* for insulin-dependent glucose transporter ($p < 0.05$, 2-way ANOVA in both cases, see Fig. 4, *b*), pointing to elevated insulin sensitivity in muscles.

Metabolic indices

Females had lower liver weights than males ($p = 0.051$, $F_{1.18} = 4.35$, 2-way ANOVA) and lower blood cholesterol levels ($p < 0.001$, $F_{1.18} = 49.82$, 2-way ANOVA). They did not differ from males in blood glucose or triglyceride levels (see the Table). Leptin administration to pregnant mothers did not affect the metabolic indices tested in either males or females.

Discussion

Previous works with mice and rats demonstrated that an increase in maternal blood leptin level increased the resistance to diet-induced obesity in the offspring, and the programming action of maternal leptin might depend on the offspring sex (Stocker et al., 2007; Makarova et al., 2013). Here, we tested the hypothesis that the said effect of maternal leptin was associated with its influence on taste preferences and on gene expression in the liver and muscles. For this purpose, we administered leptin to females at the minimum threshold dose inducing physiological response (Enriori et al., 2007) on days 11, 12, and 13 of pregnancy and assessed taste preferences, metabolic indices, and gene expression in the liver and muscles in the offspring of both sexes. We chose this time interval of pregnancy because sex differentiation in mouse fetuses starts on days 11, 12 (Hacker et al., 1995). Just on day 12, the proliferation of precursors of hypothalamic neurons that will control energy consumption and expenditure reaches its maximum (Ishii, Bouret, 2012).

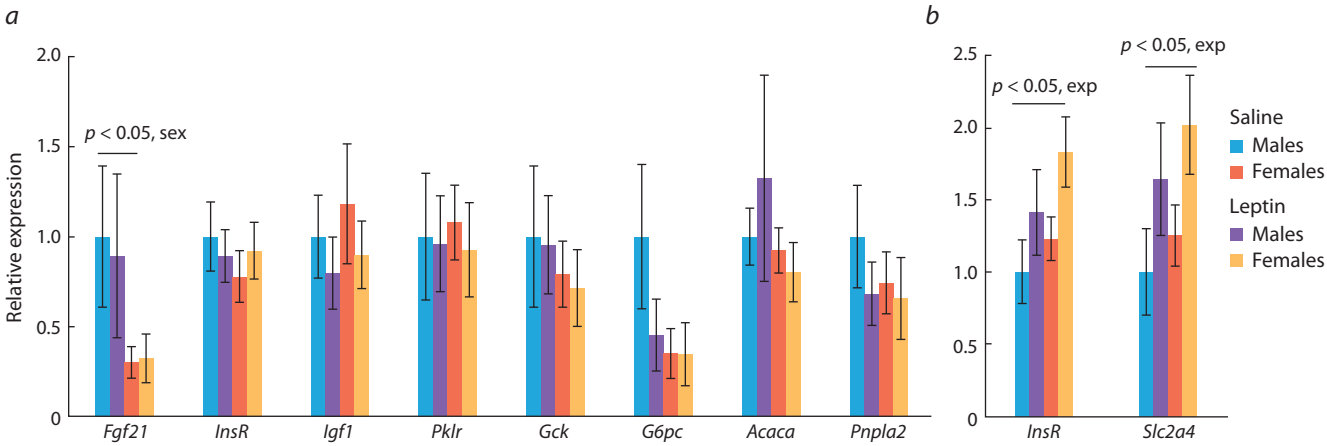

Fig. 4. Influence of leptin administered to pregnant mice on gene expression in the liver (*a*) and muscles (*b*) of the male and female offspring fed on the obesogenic diet.

Influence of leptin administration to pregnant mice on liver weight and blood biochemistry in male and female offspring fed on the obesogenic diet

Index	Males		Females	
	Control	Leptin	Control	Leptin
Cholesterol, mM	8.16 ± 1.18	8.07 ± 0.69	4.27 ± 0.43	3.55 ± 0.19
Glucose, mM	15.47 ± 2.80	11.13 ± 1.23	10.42 ± 1.10	11.99 ± 1.43
Triglycerides, mM	0.34 ± 0.07	0.50 ± 0.13	0.37 ± 0.07	0.20 ± 0.04
Liver weight, g	1.39 ± 0.03	1.42 ± 0.02	1.35 ± 0.05	1.26 ± 0.05

Cholesterol, $p < 0.001$, males *vs* females, 2-way ANOVA.

Earlier we found that a single leptin administration to mice on pregnancy day 12 exerts sex-specific programming action on metabolism in the offspring (Denisova et al., 2020). Thus, females are sensitive to leptin in this period, as evident from lower food consumption in response to leptin. At later pregnancy stages, leptin sensitivity in females may decrease as a result of the significant increase in endogenous blood leptin in the third trimester of pregnancy (Makarova et al., 2010).

Leptin administration to females slowed down the development of diet-induced obesity in the male offspring, which agrees with earlier studies, where leptin administration to female rats from day 14 of pregnancy till the end of lactation (Stocker et al., 2007) and to mice at the end of pregnancy (Makarova et al., 2013) prevented diet-induced obesity in the male offspring fed on obesogenic diet. A single injection of leptin on day 12 of pregnancy prevented hyperglycemia in the offspring with obesity and tended to decrease the rate of diet-induced obesity development in the male offspring (Denisova et al., 2020). However, in contrast to the data of this work, a single leptin administration to female mice on day 12 of pregnancy did not affect the growth rate of the female offspring fed on standard chow. Probably, the leptin administration on day 12 only left intact the initial steps of sex differentiation, which might be leptin-sensitive.

This study shows that maternal leptin may affect taste preferences in the offspring, and this effect may be one of the factors causing resistance to diet-induced obesity in the male pups with free choice of components of obesogenic diet. This result is new. Females consumed more cookies and less standard chow than males. This observation is consistent with the sex differences in consuming sweet food observed in various species (Valenstein et al., 1967; Zucker et al., 1972; Buczek et al., 2020). The causes of these differences are sought in the influence of biological sex on central systems regulating energy homeostasis and on reward systems (Sinclair et al., 2017; Buczek et al., 2020).

Our results are the first to demonstrate explicitly that the liver hormone FGF21 may be involved in the sex-dependent regulation of taste preferences. The expression of *Fgf21* in the livers of females was significantly lower than in males, which is in agreement with earlier data (Bazhan et al., 2019). The blood FGF21 level correlates with the expression rate of its gene in the liver. It is elevated in obese males (Bazhan et al., 2019). It has been shown that FGF21 increases protein consumption (Larson et al., 2019) and decreases sugar consumption (Talukdar et al., 2016); thus, the higher FGF21 level in males as compared to females may be the cause of higher consumption of standard protein-rich chow and lower consumption of sweet cookies in males than in females.

Leptin administration to pregnant females decreased cookie consumption and increased standard chow consumption in the offspring. Similar results were obtained in experiments by K.E. Pollock et al. (2015) with mice. They found that hyperleptinemia in pregnant females shifted taste preferences in the offspring towards higher consumption of standard chow as compared to sweet food.

The mechanisms underlying this effect are unknown, and our work indicates that they are not associated with *Fgf21* expression in the liver, because leptin administration to pregnant mice did not change the *Fgf21* mRNA level in the offspring. It is conceivable that the programming action of maternal leptin is associated with its influence on the motivation and reward systems and systems regulating food behavior in the offspring.

It has been shown that maternal environment factors during pregnancy and nursing may affect the motivation and reward systems in the offspring, which involve endogenous opioids, dopamine, and serotonin (Grissom et al., 2014). The type of leptin influence on the development of food consumption regulation systems demands further studies, and our results indicate that the embryo development period from day 11 to 13 is the window in which these systems are susceptible to maternal environment factors.

O.O. Talton et al. (2016) showed that high blood leptin in pregnant mice increased insulin sensitivity in the offspring regardless of diet. We found no effect of maternal leptin on the expression of liver genes involved in glucose metabolism (*InsR*, *Igf1*), fatty acid oxidation (*Fgf21*), glycolysis (*Gck*), or gluconeogenesis (*Pklr*, *G6pc*). Neither did it affect the expression of genes for lipolysis (*Atgl*) or lipogenesis (*Acaca*). Our results demonstrate that the effect of maternal leptin increasing insulin sensitivity may be mediated by the expression of genes regulating glucose metabolism in muscles of the offspring. The expression of genes for insulin receptor (*Insr*) and insulin-dependent glucose transporter (*Slc2a4*) in muscles was higher in the offspring of leptin-receiving females as compared to control ones. Apparently, maternal leptin supports insulin sensitivity in the consumption of obesogenic food by the offspring, which may also work against obesity development.

The molecular mechanisms underlying the programming action of maternal leptin are obscure. It is unknown whether maternal leptin penetrates to the fetus bloodstream through

placenta. However, at high leptin levels in pregnant females it may penetrate through placenta, as we found in our previous studies that the blood plasma leptin levels increased manifold in both the females and the fetuses within one hour after its administration at the end of pregnancy (Denisova, Makarova, 2018). Also, it has been shown that leptin administration to pregnant mice reduces the weights of fetuses and placentae (Yamashita et al., 2001; Denisova et al., 2020). The placentae of male and female fetuses respond to leptin administration differently: in male fetuses, leptin reduces placenta weight and in female ones, the expression of glucose transporters in placentae (Denisova et al., 2020). The sex-specific programming action of maternal leptin on metabolism in the offspring may be mediated by its different effects on placenta functions in fetuses of different sexes. More studies are demanded for understanding the mechanisms by which maternal leptin affects fetus development.

Conclusion

We show that triple leptin administration to females on pregnancy days 11, 12, and 13 delayed diet-induced obesity development in the male offspring. It also shifted taste preferences towards the consumption of balanced diet and increased the expression rates of genes for insulin receptor and insulin-dependent glucose transporter in muscles in the offspring of both sexes. These results suggest that maternal leptin increases the resistance to diet-induced obesity in the offspring via taste preferences and higher muscle sensitivity to insulin.

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Genotyping of potato samples from the GenAgro ICG SB RAS collection using DNA markers of genes conferring resistance to phytopathogens

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Abstract. Wart (a disease caused by *Synchytrium endobioticum*) and golden cyst potato nematode (*Globodera rostochiensis*), which parasitize the roots of the host plant, cause significant damage to potato crop. Both of these disease factors are quarantined in the Russian Federation, and each registered variety is tested for resistance to their most common races and pathotypes. The main method of opposing such diseases is by the development of resistant varieties. An important step in this process is the selection of resistant genotypes from the population and the estimation of the resistance of hybrids obtained by crosses during the breeding process. Conducting a permanent phenotypic evaluation is associated with difficulties, for example, it is not always possible to work with pathogens, and phenotypic evaluation is very costly and time consuming. However, the use of DNA markers linked to resistance genes can significantly speed up and reduce the cost of the breeding process. The aim of the study was to screen the GenAgro potato collection of ICG SB RAS using known diagnostic PCR markers linked to golden potato cyst nematode and wart resistance. Genotyping was carried out on 73 potato samples using three DNA markers 57R, CP113, Gro1-4 associated with nematode resistance and one marker, NL25, associated with wart resistance. The genotyping data were compared with the data on the resistance of the collection samples. Only the 57R marker had a high level of correlation (Spearman R = 0.722008, $p = 0.000000$, $p < 0.05$) between resistance and the presence of a diagnostic fragment. The diagnostic efficiency of the 57R marker was 86.11 %. This marker can be successfully used for screening a collection, searching for resistant genotypes and marker-assisted selection. The other markers showed a low correlation between the presence of the DNA marker and resistance. The diagnostic efficiency of the CP113 marker was only 44.44 %. Spearman's correlation coefficient (Spearman R = –0.109218, $p = 0.361104$, $p < 0.05$) did not show significant correlation between resistance and the DNA marker. The diagnostic efficiency of the NL25 marker was 61.11 %. No significant correlation was found between the NL25 marker and resistance (Spearman R = –0.017946, $p = 0.881061$, $p < 0.05$). The use of these markers for the search for resistant samples is not advisable.
Key words: golden potato cyst nematode; wart; potato; DNA markers 57R; NL25; CP113; Gro1-4.

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Генотипирование образцов картофеля коллекции «ГенАгро» ИЦиГ СО РАН с применением ДНК-маркеров генов устойчивости к фитопатогенам

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Аннотация. Значительный ущерб урожаю картофеля наносят рак картофеля (болезнь, вызываемая патогенным грибом *Synchytrium endobioticum*) и золотистая картофельная нематода (*Globodera rostochiensis*), паразитирующая на корнях растения-хозяина. Оба этих фактора являются объектами внешнего и внутреннего карантина в Российской Федерации, и каждый сорт, регистрируемый в РФ, проходит проверку на устойчивость к наиболее распространенным их расам и патотипам. Основной метод борьбы с подобными заболеваниями – выведение

устойчивых сортов. Важным этапом в этом процессе является отбор устойчивых генотипов из популяции и оценка устойчивости гибридов, полученных при скрещиваниях во время селекционного процесса. Проведение постоянной фенотипической оценки связано с рядом трудностей, а именно: не всегда есть возможность работать с патогенами, сама фенотипическая оценка очень затратная и трудоемкая. Однако применение ДНК-маркеров, сцепленных с генами устойчивости, может значительно ускорить и удешевить процесс. Целью исследования было проведение скрининга коллекции картофеля «ГенАгро» (ИЦиГ СО РАН) с использованием ПЦР-маркеров, разработанных для диагностики устойчивости к золотистой картофельной нематоде и раку картофеля. Семьдесят три образца из коллекции «ГенАгро» ИЦиГ СО РАН были генотипированы ДНК-маркерами 57R, CP113, Gro1-4, сцепленными с устойчивостью к нематоде, и маркером NL25 для устойчивости к раку. Результаты генотипирования сопоставлены с уровнем восприимчивости образцов к болезням. Высокий уровень корреляции (коэффициент корреляции Спирмена Spearman R = 0.722008, $p = 0.000000$, $p < 0.05$) между устойчивостью и наличием диагностического фрагмента был показан только для маркера 57R. Диагностическая эффективность маркера 57R составила 86.11 %. Данный маркер можно успешно использовать для поиска устойчивых генотипов и проведения маркер-ориентированной селекции. Для остальных маркеров достоверных корреляций не выявлено. Диагностическая эффективность применения маркера CP113 равнялась всего 44.44 %, а коэффициент корреляции Спирмена (Spearman R = –0.109218, $p = 0.361104$, $p < 0.05$) показывал отсутствие значимой корреляция между устойчивостью и ДНК-маркером. Диагностическая эффективность маркера NL25 составила 61.11 %. Значимой корреляции между маркером NL25 и устойчивостью не обнаружено (Spearman R = –0.017946, $p = 0.881061$, $p < 0.05$). Использование этих маркеров для поиска устойчивых образцов нецелесообразно.

Ключевые слова: золотистая картофельная нематода; рак картофеля; картофель; ДНК-маркеры 57R; NL25; CP113; Gro1-4.

Introduction

Potato is one of the most important crops in the world and is the world's fifth largest staple food crop by volume (FAO Statistical Pocketbook, 2019). One of the possible reasons for a decrease in yield is the damage of potatoes by various factors. Especially dangerous for potatoes are golden potato cyst nematode (*Globodera rostochiensis*) and potato wart (pathogen – *Synchytrium endobioticum*). They are quarantined in the Russian Federation. Data on resistance to *G. rostochiensis* and *S. endobioticum* are required when registering a potato variety in the State Register of Selection Achievements Authorized for Use (State Register… 2019; https://gossortrf.ru/).

Potato cyst nematode (PCN) can cause significant damage to the potato yield, which can reach 80–90 % (Khiutti et al., 2017; Klimenko et al., 2017). Today, 5 pathotypes of this pest are known in the world: Ro1, Ro2, Ro3, Ro4, Ro5 (Kort et al., 1977; Khiutti et al., 2017), while in Russia only the Ro1 pathotype of PCN has been detected at the moment (Limantseva et al., 2014).

Potato wart affects from 35 (Koretsky, 1970) to 100 % (Hampson, 1993) of the yield. There are 43 wart pathogens in Europe today (Baayen et al., 2006). Only a few varieties affected by this disease are registered in the State Register of Selection Achievements (State Register… 2019; https://gossortrf.ru/).

One of the main methods of dealing with these pests is the development of resistant potato varieties. Accordingly, it is important to detect genes responsible for resistance to PCN, study their heritability, develop DNA markers linked to these genes, and use genes in breeding in marker-assisted selection schemes.

The potato has 7 loci of resistance to PCN on chromosomes III (*Gro1.4*-QTL (Kreike et al., 1996)), V (*Grp1*-QTL (Rouppe van der Voort et al., 1998), *H1* (Gebhardt et al., 1993), *GroV1* (Pineda et al., 1993)), VII (*Gro1* (Barone et al., 1990; Leister et al., 1996)), X (*Gro1.2*-QTL (Kreike et al., 1993)), XI (*Gro1.3*-QTL (Kreike et al., 1993)). Four loci (*Gro1.4*, *Grp1*, *Gro1.2*, and *Gro1.3*) provide partial resistance, while three others (*H1*, *GroV1*, and *Gro1*) give high resistance to one or more pathotypes (Gebhardt, Valkonen, 2001; Bakker et al., 2004; Ramakrishnan et al., 2015). DNA markers have made it possible to identify complex loci containing several *R*-genes, including a locus containing two genes (*H1*, *GroV1*) for PCN resistance, which was identified on chromosome V in two different potato species (Gebhardt, Valkonen, 2001).

The *H1* resistance gene is introgressed into breeding varieties from *Solanum tuberosum* ssp. *andigenum* and *S. vernei* (Toxopeus, Huijsman, 1953). This gene is dominant and determines resistance to pathotypes Ro1 and Ro4 of *G. rostochiensis* (Jones et al., 1981; Gebhardt, Valkonen, 2001; Bakker et al., 2004); according to other data, it determines resistance to pathotypes Ro5 and Ro6 (Pajerowska-Mukhtar et al., 2009; Milczarek et al., 2011; Lopez-Pardo et al., 2013; Ramakrishnan et al., 2015). This gene is located at the distal part of the long arm of the V chromosome (Gebhardt et al., 1993; Pineda et al., 1993) and encodes the CC-NBS-LRR protein (coiled coil/nucleotide-binding/leucine-rich repeat). The *H1* gene is the only nematode resistance gene for which Flora's gene-to-gene interaction concept has been validated by classical genetic analysis (Flor, 1971; Janssen et al., 1991; Gebhardt, Valkonen, 2001). The *H1* resistance gene corresponded to the *Avr* gene of golden potato cyst nematode *G. rostochiensis*.

The *GroV1* gene originates from the wild potato species *S. vernei*, is linked to the *H1* locus (Jacobs et al., 1996), and is responsible for resistance to the Ro1 pathotype of *G. rostochiensis* (Jacobs et al., 1996; Milczarek et al., 2011; Ramakrishnan et al., 2015).

The *Gro1* locus is localized on chromosome VII and contains a family of genes *Gro1-1*, *Gro1-2*, *Gro1-3*, *Gro1-4*, *Gro1-5*, *Gro1-6*, *Gro1-8*, *Gro1-10*, *Gro1-11*, *Gro1-12* and *Gro1-14*, as well as a number of pseudogenes (Barone et al., 1990; Leister et al., 1996; Paal et al., 2004). J. Paal and colleagues showed that the *Gro1-4* gene is a monogenic dominant gene responsible for resistance to the Ro1 pathotype of *G. rostochiensis* and encodes a protein belonging to the TIR-NB-LRR class of proteins. *Gro1-4* was introduced into

S. tuberosum from the wild potato *S. spegazzinii* (Ballvora et al., 1995; Gebhardt, Valkonen, 2001; Gebhardt et al., 2004; Paal et al., 2004; Kuhl, 2011; Milczarek et al., 2011; Ramakrishnan et al., 2015).

A number of loci of quantitative traits associated with resistance to cyst nematodes were mapped in the potato genome: *Gro1.2*, *Gro1.3*, and *Gro1.4* determining resistance to *G. rostochiensis* were localized on chromosomes X, XI, and III. In this case, *S. spegazzinii* was the source of resistance (Kreike et al., 1993, 1996).

The *Grp1* locus provides a broad spectrum of resistance to both cyst nematodes *G. rostochiensis* and *G. pallida*. It has been mapped to chromosome V (Rouppe van der Voort et al., 1998, 2000) and determines resistance to the Ro5 pathotype of *G. rostochiensis* (Finkers-Tomczak et al., 2009; Milczarek et al., 2011; Ramakrishnan et al., 2015).

A significant number of diagnostic DNA markers have been developed for the *H1* gene. Among them are markers CD78 (Pineda et al., 1993), TG689 (Milczarek et al., 2011; Lopez-Pardo et al., 2013), N146, N195 (Mori et al., 2011; Asano et al., 2012), CP113 (Gebhardt et al., 1993; Niewöhner et al., 1995; Skupinová et al., 2002; Milczarek et al., 2011), TG689/TG689indel12 (Galek et al., 2011), 239E4left (Bakker et al., 2004; Pajerowska-Mukhtar et al., 2009; Milczarek et al., 2011), EM15 (repulsion) and CMI (coupling) (Bakker et al., 2004), 57R (Finkers-Tomczak et al., 2011; Schultz et al., 2012; Milczarek et al., 2014). Markers have also been designed for other genes and QTLs. For example, markers TG69 (Pineda et al., 1993), SCAR-U14, and SCAR-X02 have been developed for the *GroV1* gene (Jacobs et al., 1996; Milczarek et al., 2011); markers CP56 and St3.3.2 (Barone et al., 1990; Leister et al., 1996), CP56, CP51(c), GP516(c) were selected for the *Gro1* locus (Ballvora et al., 1995; Kuhl, 2011). Markers Gro1-4 (Gebhardt et al., 2004; Paal et al., 2004; Milczarek et al., 2011) and Gro1-4-1 (Asano et al., 2012) were designed for the *Gro1-4* gene. For *Grp1*-QTL, markers GP21 and GP179 (Rouppe van der Voort et al., 1998), TG432 (Finkers-Tomczak et al., 2009; Milczarek et al., 2011) have been developed. The TG63 marker was selected for *Gro1.2*-QTL (Kreike et al., 1993). Markers Ssp75 and TG30 have been developed for *Gro1.3*-QTL (Kreike et al., 1993). The Ssp8 marker was designed for *Gro1.4*-QTL (Kreike et al., 1996).

A number of genes for resistance to wart (*S. endobioticum*) have been found in potatoes. These are the following genes: *Sen1*, located on the XI chromosome (Hehl et al., 1999); *Sen1-4* mapped to chromosome IV (Brugmans et al., 2006); locus *Sen18*-IX, located on chromosome IX; locus *Sen2*/*6*/*18*-I, located on chromosome I (Ballvora et al., 2011); locus *Xla-TNL* found on chromosome XI (Bartkiewicz et al., 2018); the *Sen2* locus mapped to chromosome XI (Plich et al., 2018); the *Sen3* locus was mapped on chromosome XI in the same region as the *Sen1* gene (Prodhomme et al., 2019); the authors suggested that *Sen3* could be either a *Sen1* paralogue from the same cluster or an allelic variant of the *Sen1* gene.

QTLs responsible for resistance to races 1, 2, 6 and 18 of wart are found on other chromosomes: chromosome I (to race 2), chromosome II (to races 6, 18), chromosome VI (to races 1, 2, 6, 18), chromosome VII (to races 2, 6, 18), chromosome VIII (to races 1, 2, 6, 18), chromosome X (to races 2, 6, 18), chromosome XI (to races 2, 6, 18) (Groth et al., 2013). J.E. Obidiegwu and colleagues also found additional wart resistance loci on chromosomes I, IV, X, XI, and XII that were less influential than the main genes (Obidiegwu et al., 2015). Minor QTLs located on the chromosome X further affect resistance to race 18 of wart (Bartkiewicz et al., 2018).

The *Sen1* and *Sen1-4* genes determine the resistance to race 1 of the potato wart pathogen; in both cases, resistance is determined by the dominant alleles of the genes. The *Sen1* gene is located at the distal part of the long arm of chromosome XI (Hehl et al., 1999; Obidiegwu et al., 2014). However, it should be noted that J.E. Obidiegwu et al. (2015), using genome-wide association studies (GWAS), identified the *Sen1*/*RSe*-XIa multi-allelic locus on potato chromosome XI as the main factor of resistance to four *S. endobioticum* races (races 1, 2, 6 and 18) (Obidiegwu et al., 2015). The *Sen1-4* gene is located on the long arm of chromosome IV at a distance of 5 cM from the centromere (Brugmans et al., 2006).

The *Xla-TNL* locus on potato chromosome XI is linked to resistance to races 18 and 6 and can be considered as one of the main factors of wart resistance (Bartkiewicz et al., 2018).

The *Sen2* locus is mapped to chromosome XI and is a dominant monogenic locus that provides a high level of resistance to eight races of *S. endobioticum* simultaneously: 1 (D1), 2 (G1), 6 (O1), 8 (F1), 18 (T1), 2 (Ch1), 3 (M1) and 39 (P1). The genetic and physical distances between the *Sen1* and *Sen2* loci were indirectly estimated at 63 cM and 32 Mbp, respectively (Plich et al., 2018).

Sen3 is a dominant monogenic locus of resistance to races 2, 6, and 18 (Prodhomme et al., 2019). Locus *Sen18*-IX (chromosome IX) determines resistance to race 18 *S. endobioticum*, and locus *Sen2*/*6*/*18*-I (chromosome I) to races 2, 6, and 18. A. Ballvora et al. (2011) note that resistances to races 2, 6 and 18 correlate with each other, but are inherited regardless of resistance to race 1.

Several markers have been developed to detect the dominant allele of the *Sen1* gene: CP58, GP125 (Hehl et al., 1999), NL25 (Hehl et al., 1999; Bormann et al., 2004; Gebhardt et al., 2006), Sti046, St_At5g16710, GP125 and GP259 (Ballvora et al., 2011). Also, using a genome-wide association studies, a haplotype-specific marker PotVar0067008 associated with *Sen1* was identified (Prodhomme et al., 2020).

To identify the *Sen18*-IX locus, markers GP129, GP101 and STM3023b can be used. The *Sen2*/*6*/*18*-I locus can be diagnosed using markers STM2030, SC176, GP192, GP124, and GP194 (Ballvora et al., 2011). Markers Kc8103 and RK36, located on chromosome XI and linked to the *Xla-TNL* locus, have shown potential diagnostic value in determining resistance to races 18 and 6 of *S. endobioticum* (Bartkiewicz et al., 2018). Three markers, 5450_3, 2502_1, and 2502_3, linked to the *Sen2* locus were developed (Plich et al., 2018). It is possible to use the markers chr11_1259552 and chr11_1772869 to detect *Sen3* (Prodhomme et al., 2019).

The aim of the study was to screen the GenAgro potato collection of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (ICG SB RAS) using known diagnostic PCR markers linked to resistance to golden cyst potato nematode and potato wart.

Materials and methods

Plant material. The research material was the collection of varieties and hybrids of potatoes named the "GenAgro" plant collection of the ICG SB RAS. The collection was represented by 73 varieties and hybrids of potatoes (*Solanum tuberosum*) (Supplement 1)[1]. The plants were grown in the field on the territory of the Michurinsky village, Novosibirsk region, from May to August 2017.

Field tests were carried out according to the following scheme: the number of rows for each genotype was two; the number of plants in a row – 10; row length – 3 m; distance between the rows – 0.75 m; distance between the plants in rows – 0.30 m; planting method – manually (by hand) on furrows, filling furrows with harrows; landing date is the third decade of May.

Agrochemical characteristics of the soil: the content of exchanged potassium 110.00 mg/kg; the amount of exchanged bases 24.19 mg-eq/100 g; hydrolytic acidity 3.23 mg-eq/100 g; exchanged acidity 5.60 mg-eq/100 g; humus content 2.67 %; the content of mobile phosphorus 5.14 mg/kg; the degree of saturation with bases (V) 88.20 %.

Most of the data on resistance to PCN and potato wart were taken from references, namely from the database of the State Register of Selection Achievements Authorized for Use (State Register…, 2019; https://gossortrf.ru/), and from the European Cultivated Potato Database (https://www.europotato.org/). Some of the samples and hybrids for which there were no published data on resistance were evaluated under experimental conditions. Determination of resistance to PCN was carried out in accordance with the methodology recommended by OEPP/EPPO (2006) at the All-Russian Institute of Plant Protection. Potato wart resistance was evaluated according to the Glynn–Lemmerzahl method as described in the EPPO Diagnostic protocol for *S. endobioticum* (OEPP/EPPO, 2004) at the Russian Potato Research Center.

DNA isolation and PCR analysis. DNA was isolated from the skin of potato tubers using the DNeasy Plant Mini kit (Qiagen, CA, USA) according to the protocol. The concentration and purity of the tested samples were determined using gel electrophoresis and a Nanodrop 2000 apparatus.

Several diagnostic markers most often used in breeding programs were selected for genotyping (Table 1). These markers were associated with *R*-genes that determine resistance to race 1 of potato wart (*S. endobioticum*) and Ro1 pathotype of potato cyst nematode (*G. rostochiensis*).

Two markers, 57R and CP113, associated with the *H1* resistance gene, and the Gro1-4 marker, associated with the *Gro1-4* resistance gene, were selected to identify PCN resistance genes (see Table 1). The SCAR PCR marker CP113-5′2/CP113-3′2 was proposed by J. Niewöhner et al. (1995) based on the RFLP marker CP113. Amplification of DNA of resistant genotypes using this marker formed product with a 760 bp length. The 57R marker was proposed by L. Schultz et al. (2012). Amplification of DNA of resistant genotypes formed product with a 450 bp length. SCAR PCR marker Gro1-4 was developed by J. Paal et al. (2004) based on the RFLP marker Gro1. Amplification of DNA of resistant genotypes formed product with a 602 bp length.

[1] Supplementary Materials are available in the online version of the paper: http://vavilov.elpub.ru/jour/manager/files/Suppl_Totsky_Engl.pdf

The NL25 marker was proposed by R. Hehl et al. (1999) when mapping the *Sen1* gene. C.A. Bormann et al. (2004) and C. Gebhardt et al. (2006) used this marker for marker-assisted selection (see Table 1). Amplification produces one or two fragments of 1200 or 1400 bp lenght. The presence of the dominant *Sen1* allele is determined by the presence of a 1400 bp fragment.

PCR was carried out in a 20 μL reaction mixture containing 100 ng of DNA, 67 mM Tris-HCl (pH 8.8), 1.8 mM $MgCl_2$, 0.01 % Tween 20, 0.2 mM each dNTP, 0.25 μM forward and reverse specific primers, 1 unit Taq DNA polymerase.

Two types of amplification programs (SSR55 and SSR60) represented the time-temperature profile of PCR. SSR55: (1) first cycle: 94 °C – 2 min; (2) the next 45 cycles: 94 °C – 1 minute, 55 °C – 1 minute and 72 °C – 2 minutes; (3) one cycle of 5 minutes at 72 °C (Gro1-4). SSR60: (1) first cycle: 94 °C – 2 min; (2) the next 45 cycles: 94 °C – 1 minute, 60 °C – 1 minute and 72 °C – 2 minutes; (3) one cycle of 5 minutes at 72 °C (NL25, CP113, 57R).

The analysis of the obtained PCR products was carried out by electrophoresis in a 2 % agarose gel. The results were documented using a Molecular Imager Gel Doc XR System (BioRad) using UV light.

Statistical processing of the data was carried out using Spearman's correlation coefficient; for calculations, the STATISTICA program was used. The diagnostic efficiency, sensitivity, specificity and predictive value were calculated using the MedCalc software (https://www.medcalc.org/). Diagnostic efficiency was defined as the proportion of correct test results in the total number of test results, or the sum of true positive and true negative test results divided by the total number of test results. The sensitivity was calculated as the number of resistant samples identified using a DNA marker divided by the total number of resistant samples. Specificity is the number of susceptible samples identified by the DNA marker divided by the total number of susceptible samples. Positive predictive value was defined as the proportion of correct positive diagnostic test results.

Results

Among 73 samples selected for genotyping, 35 were resistant to PCN, 37 samples were susceptible, and in one sample, resistance to nematodes was unknown (Table 2). 69 samples were resistant to wart, 3 samples were susceptible to disease, the resistance of one sample was unknown (see Table 2).

Genotyping of varieties and hybrids using markers designed for resistance to PCN

The 57R marker is found in 85.7 % of resistant samples, as well as in 13.5 % of susceptible ones (Table 3; Supplement 2, Fig. 1–6; Supplement 3). Some mismatches can be observed due to the absence of linkage of the 57R marker with the *H1* resistance gene in a number of samples. The second reason for the mismatches can be explained by the presence of other resistance genes in samples that do not carry the 57R marker. The diagnostic efficiency of the 57R marker, which is expressed as the percentage of true (both positive and negative) test results to the total number of results obtained, was 86.11 %. The diagnostic sensitivity of the used marker, which shows the number of resistant samples identified using the DNA marker

Table 1. DNA markers used for collection screening

Gene	Trait	Marker and primer name	Nucleotide sequence from 5′ to 3′	Gene localization	Diagnostic fragment size, bp	Reference
H1	Resistance to golden potato cyst nematode (*G. rostochiensis*)	CP113F	GCGTTACAGTCGCCGTAT	Chromosome V	760	Niewöhner et al., 1995
		CP113R	GTTGAAGAAATATGGAATCAAA			
		57R-F	TGCCTGCCTCTCCGATTTCT		450	Schultz et al., 2012
		57R-R	GGTTCAGCAAAAGCAAGGACGTG			
Gro1-4		Gro1-4F	TCTTTGGAGATACTGATTCTCA	Chromosome VII	602	Paal et al., 2004
		Gro1-4R	CGACCTAAAATGAAAAGCATCT			
Sen1	Resistance to wart (pathogen – *S. endobioticum*)	NL25F	TATTGTTAATCGTTACTCCCTC	Chromosome XI	1400	Hehl et al., 1999; Bormann et al., 2004; Gebhardt et al., 2006
		NL25R	AGAGTCGTTTTACCGACTCC			

divided by the total number of resistant samples, was 85.71 %. The diagnostic specificity, which is the number of susceptible samples identified by the DNA marker divided by the total number of susceptible samples, was 86.48 %. The predictive value of a positive result, showing the proportion of correct positive diagnostic test results, was 85.71 %. Calculation of the Spearman correlation coefficient (Spearman R = 0.722008, $p = 0.000000$, $p < 0.05$) showed a significant correlation between resistance and the 57R marker.

The CP113 marker is found in only 48.6 % of resistant accessions, while the marker is present in 62.9 % of susceptible genotypes (see Table 3; Supplement 2, Fig. 7; Supplement 3). These results can be regarded as the absence of linkage of the marker with the *H1* resistance gene in many samples of the potato collection. The diagnostic efficiency of the CP113 marker was only 44.44 %. Diagnostic sensitivity was 48.57 %. Diagnostic specificity accounted for 40.54 %. The predictive value of a positive result, indicating the probability of resistance presence if the test shows a positive result when CP113 marker was used, was equal to 43.58 %. Spearman's correlation coefficient (Spearman R = –0.109218, $p = 0.361104$, $p < 0.05$) in this case showed no significant correlation between resistance and DNA marker. The use of such a marker when screening a population to search for resistant samples is not advisable.

29 samples were analyzed using the Gro1-4 marker. The diagnostic fragment was amplified in only 5 samples. Correspondence of the presence of the marker in the resistant sample was observed only in 1 case out of 5. In other cases, the marker was found in the samples susceptible to the disease.

The data obtained show that when screening populations for resistance to PCN, it is advisable to use the 57R marker.

Genotyping of varieties and hybrids using markers linked to resistance to potato wart

The NL25 marker is found in 62.3 % of resistant samples, however, the marker is present in two of the three susceptible genotypes (see Table 3, Supplement 4). This can be explained by the processes of crossing over and by the fact that in a number of samples the linkage of the marker and the resistance gene is not observed; however, the small number of sensitive samples does not allow sufficiently assessing the applicability of the marker for breeding. The marker is absent in 27 samples and only in one case we observe the absence of a marker in the susceptible sample, in the other cases the marker is absent in the resistant samples. This can be explained by the presence of another resistance gene that is not linked to the NL25 marker.

The diagnostic efficiency of resistance using the NL25 marker was 61.11 %. The diagnostic sensitivity turned out to be at 62.31 %. The diagnostic specificity was only 33.33 %. However, the predictive value of a positive result, showing the proportion of correct positive diagnostic test results, when using the NL25 marker was equal to 95.55 %. It should be noted that such results are associated with the fact that the set of samples contained only three sensitive samples, and two of them showed the presence of the NL25 marker. Spearman's correlation coefficient (Spearman R = –0.017946, $p = 0.881061$, $p < 0.05$) in such situation showed the absence of significant correlations.

Despite the fact that the NL25 marker is often used in screening and marker selection, a study in our set of samples showed that its use does not guarantee a reliable result.

Discussion

In our study, 13 resistant to golden potato nematode samples that had both markers (57R and CP113) linked to the *H1* nematode resistance gene were found. In addition, there are 8 genotypes resistant to nematodes and wart and carrying both the 57R and CP113 markers linked to the *H1* nematode resistance gene and the NL25 marker linked to the *Sen1* wart resistance gene. There is also one sample (Safo) in the population that is resistant to wart and nematodes and carries all three markers 57R, CP113, Gro1-4, linked to nematode resistance, and marker NL25, linked to wart resistance.

DNA markers of wart resistance

The NL25 marker linked to the *Sen1* gene, which provides resistance to pathotype 1 of potato wart, is successfully used in the practice of marker-oriented selection. So, C. Gebhardt and colleagues reported that after screening 17 plants in two families of segregating populations using the NL25 marker,

Table 2. Resistance of varieties and hybrids of potatoes to nematodes and wart

No.	Variety/hybrid	Resistance to golden potato cyst nematode	Resistance to wart	No.	Variety/hybrid	Resistance to golden potato cyst nematode	Resistance to wart
1	Fregata	+[2]	+[2]	38	Lomonosovskij	–[1]	+[1]
2	Agata	+[2]	+[2]	39	Lyubava	–[1]	+[1]
3	Alyona	–[1]	+[1]	40	Lyuks	+[1]	+[1]
4	Antonina	–[1]	+[1]	41	Maret	+[1]	NA
5	Aroza	+[1]	+[1]	42	Matushka	–[1]	+[1]
6	Bozhedar	NA	+[1]	43	Meteor	+[1]	+[1]
7	Bravo	+[1]	+[1]	44	Monaliza	–[1]	+[1]
8	Vasilyok	–[1]	+[1]	45	Nakra	–[1]	+[1]
9	Velikan	–[1]	+[1]	46	Nayada	+[1]	+[1]
10	Virazh	+[1]	+[1]	47	Nevskij	–[1]	+[1]
11	Vympel	+[1]	+[1]	48	Nikulinskij	–[1]	+[1]
12	Gala	+[1]	+[1]	49	Pamyati Osipovoj	–[1]	+[1]
13	Golubizna	–[1]	+[1]	50	Pamyati Rogachyova	–[1]	+[1]
14	Gornyak	–[1]	+[1]	51	Pikasso	+[1]	+[1]
15	Granat	–[2]	+[2]	52	Reggi	–[1]	+[1]
16	Granola	+[2]	–[2]	53	Red Skarlet	+[1]	+[1]
17	Gulliver	+[1]	+[1]	54	Safo	+[1]	+[1]
18	Gusar	+[1]	+[1]	55	Favorit	+[1]	+[1]
19	Debryansk	–[1]	+[1]	56	Fioletovyj	–[1]	+[1]
20	Diamant	+[2]	+[2]	57	Fritella	–[1]	+[1]
21	Zhigulyovskij	–[1]	+[1]	58	Yuna	+[1]	+[1]
22	Zhukovskij rannij	+[1]	+[1]	59	1-7-5A	–[3]	+[4]
23	Zagadka	+[1]	+[1]	60	(1-9-2)	–[3]	+[4]
24	Zekura	+[1]	+[1]	61	2-5-4B	–[3]	+[4]
25	Zlatka	–[1]	+[1]	62	1-14-2A	+[3]	+[4]
26	Il'inskij	–[1]	+[1]	63	1014/3-1	+[3]	+[4]
27	Impala	+[1]	+[1]	64	821/1-5	+[3]	+[4]
28	Irbitskij	+[1]	+[1]	65	419/8-1	–[3]	+[4]
29	Kemerovchanin	+[1]	+[1]	66	1014/8-1	+[3]	+[4]
30	Klada	–[2]	+[2]	67	1013/3-1	+[3]	+[4]
31	Koldovskaya	–[3]	–[4]	68	790/1-5	–[3]	+[4]
32	Kolobok	–[1]	+[1]	69	(2-5-2)	+[3]	+[4]
33	Kortni	+[1]	+[1]	70	785/8-5	–[3]	+[4]
34	Krepysh	+[1]	+[1]	71	999/1-1	–[3]	+[4]
35	Kuznechanka	–[1]	+[1]	72	597/4-1	–[3]	–[4]
36	Ladozhskij	+[1]	+[1]	73	417/2	–[3]	+[4]
37	Lina	–[1]	+[1]				

Note. [1] State Register of Selection Achievements Authorized for Use; [2] The European Cultivated Potato Database; [3] All-Russian Institute of Plant Protection; [4] Russian Potato Research Center; NA – no data.

Table 3. Results of screening of potato cultivars and hybrids collection for PCN resistance using the 57R marker and for wart resistance using the NL25 marker

Disease resistance	Marker is present	Marker is missing	Diagnostic efficiency, %
Marker 57R			
PCN resistant sample	30	5	86.11
PCN susceptible sample	5	32	
Marker CP113			
PCN resistant sample	17	18	44.44
PCN susceptible sample	22	15	
Marker NL25			
PCN resistant sample	43	26	61.11
PCN susceptible sample	2	1	

14 genotypes with the marker were identified. All these plants were found to be resistant to pathotype 1 *S. endobioticum*. Some were also resistant to pathotype 2 and/or pathotype 6 (Gebhardt et al., 2006).

The effectiveness of this marker is also reported by O.Y. Antonova and colleagues who analyzed 98 varieties using the NL25 marker. A diagnostic component was found in 95 studied wart-resistant varieties, while it was not found in three susceptible varieties. This shows a high level of correlation between the presence or absence of the marker and the resistance and sensitivity of the genotype to wart, respectively (Antonova et al., 2016).

However, A. Khiutti and colleagues, when screening 52 genotypes using the NL25 marker, found that 39 samples (both sensitive and resistant genotypes) had the same nondiagnostic fragment, 12 genotypes did not have amplification of the NL25 marker fragments. Only 5 out of 52 genotypes had a diagnostic fragment indicating the presence of a resistance gene. Four of these five accessions were resistant, but one genotype was found to be sensitive; most resistant genotypes did not have a 1400 bp diagnostic fragment predicting a resistant phenotype (Khiutti et al., 2012).

Our analysis also did not allow us to speak about the reliability of using the NL25 marker for screening resistant varieties.

DNA markers of resistance to PCN

Using the Gro1-4 marker in a segregating population, C. Gebhardt and colleagues found that all 45 plants carrying this marker linked to the *Gro1* gene were resistant to the Ro1 pathotype of *G. rostochiensis* (Gebhardt et al., 2006).

C. Gebhardt and colleagues in 1993 found in a segregating population that the CP113 marker is linked to the *H1* gene so strongly that it has zero recombination (Gebhardt et al., 1993). However, D. Milczarek and colleagues (2011) reported that the CP113 marker was amplified for all tested varieties, resistant and sensitive, and was unsuitable for the selection of resistant clones. A similar picture is observed in our work.

The 57R SCAR marker was tested in a mapping population, where it was linked to the *H1* locus and nematode resistance (Finkers-Tomczak et al., 2011). Later L. Schultz and colleagues reported that they analyzed two independent populations of 281 and 122 potato samples with known resistance/sensitivity using the 57R SCAR marker. When screening the first population, the 57R marker revealed a correspondence between genotype and phenotype, 89 out of 90 resistant varieties had an allele associated with resistance. Only one resistant variety, in which no marker amplification was observed, became an exception. None of the 191 PCN susceptible varieties had an allele predicting resistance. Then another independent population of 122 varieties was screened. All varieties showed complete correspondence between resistance to *G. rostochiensis* and the presence/absence of the 57R allele, corresponding to the presence of the resistance gene (Schultz et al., 2012).

O.Y. Antonova et al. (2016) identified the 57R marker in 33 (30.3 %) of 109 breeding varieties they studied. The overwhelming majority of the varieties with the diagnosed 57R fragment were resistant or weakly affected by the nematode. The correspondence between resistance and the presence of a diagnostic fragment was high – 93.5 %. At the same time, only four genotypes with the Gro1-4 marker were identified: two resistant varieties, one weakly affected variety and one susceptible. All these four varieties, along with the Gro1-4 marker, also possessed the *H1* gene markers – 57R, TG689, N146, N195 (Antonova et al., 2016).

In the work of N.S. Klimenko et al. (2017) showed the presence of the 57R marker in 24 out of 103 samples, while the marker was found in 15 resistant and 2 susceptible samples. It was shown that the correlation between the presence of at least one marker of the *H1* gene and the data on the nematode resistance of varieties was +0.92 (Klimenko et al., 2017).

T.A. Gavrilenko et al. (2018) showed that out of 39 samples of the studied set of samples, 15 had a dominant allele of the *H1* gene (based on a number of DNA markers), and two varieties had dominant alleles of both *H1* and *Gro1-4* genes. At the same time, none of the markers was identified in the remaining 22 genotypes. Comparison of these results with resistance to *G. rostochiensis* (pathotype Ro1) showed that all accessions with *H1* gene markers are nematode resistant, while varieties affected by *G. rostochiensis* did not have these markers (Gavrilenko et al., 2018). This high correlation shows

the reliability of the markers used in the study, which can be used to select resistant samples.

It should be noted that the saturation of the genotype with genes of resistance to the nematode does not affect its economically valuable traits. At the same time, there is a strong link between the presence of the marker and resistance. So, in the study of D. Milczarek and colleagues in 2014, the relationship between the presence of markers TG689 and 57R linked to the *H1* gene, which determines resistance to the nematode *G. rostochiensis*, and valuable agricultural traits is presented. Clones with these markers had a higher total yield of tubers and total starch yield than clones without markers. There was no negative association between marker presence and quality. All 347 seedlings obtained after three crosses were genotyped using both markers and phenotypically evaluated for resistance to the Ro1 pathotype of *G. rostochiensis*. Of these, 316 (i. e. 91 %) and 325 (94 %) clones were resistant and carried the TG689 or 57R markers (Milczarek et al., 2014).

Conclusion

In general, our data on the 57R marker are quite close to the results described above and confirm the high reliability of the work of this marker, which suggests the need to use this marker when selecting samples resistant to PCN.

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